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TITLE

NUCLEIC ACID PHARMACEUTICALS

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TITLE OF THE INVENTION

NUCLEIC ACID PHARMACEUTICALS

SUMMARY OF THE INVENTION

DNA constructs capable of being expressed upon direct introduction, via injection or otherwise, into animal tissues, are novel prophylactic pharmaceuticals. They induce cytotoxic T lymphocytes (CTLs) specific for viral antigens which respond to different strains of virus, in contrast to antibodies which are generally strain-specific. The generation of such CTLs in vivo usually requires endogenous expression of the antigen, as in the case of virus infection. To generate a viral antigen for presentation to the immune system, without the limitations of direct peptide delivery or the use of viral vectors, plasmid DNA encoding human influenza virus proteins was injected into the quadriceps of BALB/c mice, this resulted in the generation of influenza virus-specific CTLs and protection from subsequent challenge with a heterologous strain of influenza virus, as measured by decreased viral lung titers, inhibition of weight loss, and increased survival. High titer neutralizing antibodies to hemagglutinin and antibodies to nucleoprotein were generated in rhesus monkeys, and decreased nasal virus titers were observed following homologous and heterologous challenge in ferrets.

BACKGROUND OF THE INVENTION

A major challenge to the development of vaccines against viruses (such as influenza A or HIV), against which neutralizing antibodies are generated, is the diversity of the viral envelope proteins among different isolates or strains. As cytotoxic T-lymphocytes in both mice and humans are capable of recognizing epitopes derived from conserved internal viral proteins [J.W. Yewdell *et al.*, Proc. Natl. Acad. Sci. (USA) **82**, 1785 (1985); A.R.M. Townsend, *et al.*, Cell **44**, 959 (1986); A.J. McMichael *et al.*, J. Gen. Virol. **67**, 719 (1986); J. Bastin *et al.*, J. Exp. Med. **165**, 1508 (1987); A.R.M. Townsend and H. Bodmer, Annu. Rev. Immunol. **7**, 601 (1989)], and are thought to be important in the immune response against viruses [Y.-L. Lin and B.A.

Askonas, J. Exp. Med. **154**, 225 (1981); I. Gardner *et al.*, Eur. J. Immunol. **4**, 68 (1974); K.L. Yap and G.L. Ada, Nature **273**, 238 (1978); A.J. McMichael *et al.*, New Engl. J. Med. **309**, 13 (1983); P.M. Taylor and B.A. Askonas, Immunol. **58**, 417 (1986)], efforts have been directed towards the development of CTL vaccines capable of providing heterologous protection against different viral strains.

CD8+ CTLs kill virally-infected cells when their T cell receptors recognize viral peptides associated with MHC class I molecules [R.M. Zinkernagel and P.C. Doherty, *ibid.* **141**, 1427 (1975); R.N. Germain, Nature **353**, 605 (1991)]. These peptides are derived from endogenously synthesized viral proteins, regardless of the protein's location or function within the virus. Thus, by recognition of epitopes from conserved viral proteins, CTLs may provide cross-strain protection. Peptides capable of associating with MHC class I for CTL recognition originate from proteins that are present in or pass through the cytoplasm or endoplasmic reticulum [J.W. Yewdell and J.R. Bennink, Science **244**, 1072 (1989); A.R.M. Townsend *et al.*, Nature **340**, 443 (1989); J.G. Nuchtern *et al.*, *ibid.* **339**, 223 (1989)]. Therefore, in general, exogenous proteins, which enter the endosomal processing pathway (as in the case of antigens presented by MHC class II molecules), are not effective at generating CD8+ CTL responses.

Most efforts to generate CTL responses have either used replicating vectors to produce the protein antigen within the cell [J.R. Bennink *et al.*, *ibid.* **311**, 578 (1984); J.R. Bennink and J.W. Yewdell, Curr. Top. Microbiol. Immunol. **163**, 153 (1990); C.K. Stover *et al.*, Nature **351**, 456 (1991); A. Aldovini and R.A. Young, Nature **351**, 479 (1991); R. Schafer *et al.*, J. Immunol. **149**, 53 (1992); C.S. Hahn *et al.*, Proc. Natl. Acad. Sci. (USA) **89**, 2679 (1992)], or they have focused upon the introduction of peptides into the cytosol [F.R. Carbone and M.J. Bevan, J. Exp. Med. **169**, 603 (1989); K. Deres *et al.*, Nature **342**, 561 (1989); H. Takahashi *et al.*, *ibid.* **344**, 873 (1990); D.S. Collins *et al.*, J. Immunol. **148**, 3336 (1992); M.J. Newman *et al.*, *ibid.* **148**, 2357 (1992)]. Both of these approaches have limitations that may reduce their utility as vaccines. Retroviral vectors have restrictions on

the size and structure of polypeptides that can be expressed as fusion proteins while maintaining the ability of the recombinant virus to replicate [A.D. Miller, Curr. Top. Microbiol. Immunol. **158**, 1 (1992)], and the effectiveness of vectors such as vaccinia for subsequent immunizations may be compromised by immune responses against the vectors themselves [E.L. Cooney *et al*., Lancet **337**, 567 (1991)]. Also, viral vectors and modified pathogens have inherent risks that may hinder their use in humans [R.R. Redfield *et al*., New Engl. J. Med. **316**, 673 (1987); L. Mascola *et al*., Arch. Intern. Med. **149**, 1569 (1989)]. Furthermore, the selection of peptide epitopes to be presented is dependent upon the structure of an individual's MHC antigens and, therefore, peptide vaccines may have limited effectiveness due to the diversity of MHC haplotypes in outbred populations.

Benvenisty, N., and Reshef, L. [PNAS **83**, 9551-9555, (1986)] showed that $CaCl_2$ precipitated DNA introduced into mice intraperitoneally, intravenously or intramuscularly could be expressed. The intramuscular (i.m.) injection of DNA expression vectors in mice has been demonstrated to result in the uptake of DNA by the muscle cells and expression of the protein encoded by the DNA [J.A. Wolff *et al*., Science **247**, 1465 (1990); G. Ascadi *et al*., Nature **352**, 815 (1991)]. The plasmids were shown to be maintained episomally and did not replicate. Subsequently, persistent expression has been observed after i.m. injection in skeletal muscle of rats, fish and primates, and cardiac muscle of rats [H. Lin *et al*., Circulation **82**, 2217 (1990); R.N. Kitsis *et al*., Proc. Natl. Acad. Sci. (USA) **88**, 4138 (1991); E. Hansen *et al*., FEBS Lett. **290**, 73 (1991); S. Jiao *et al*., Hum. Gene Therapy **3**, 21 (1992); J.A. Wolff *et al*., Human Mol. Genet. **1**, 363 (1992)]. The technique of using nucleic acids as therapeutic agents was reported in WO90/11092 (4 October 1990), in which naked polynucleotides were used to vaccinate vertebrates.

It is not necessary for the success of the method that immunization be intramuscular. Thus, Tang et al., [Nature, **356**, 152-154 (1992)] disclosed that introduction of gold microprojectiles coated with DNA encoding bovine growth hormone (BGH) into the skin of

mice resulted in production of anti-BGH antibodies in the mice. Furth et al., [Analytical Biochemistry, **205**, 365-368, (1992)] showed that a jet injector could be used to transfect skin, muscle, fat, and mammary tissues of living animals. Various methods for introducing nucleic acids was recently reviewed by Friedman, T., [Science, **244**, 1275-1281 (1989)] See also Robinson et al., Abstracts of Papers Presented at the 1992 meeting on Modern Approaches to New Vaccines, Including Prevention of AIDS, Cold Spring Harbor, p92, where the im, ip, and iv administration of avian influenza DNA into chickens was alleged to have provided protection against lethal challenge. However, there was no disclosure of which avian influenza virus genes were used. In addition, only H7 specific immune responses were alleged, without any mention of induction of cross-strain protection.

Therefore, this invention contemplates any of the known methods for introducing nucleic acids into living tissue to induce expression of proteins. This invention provides a method for introducing viral proteins into the antigen processing pathway to generate virus-specific CTLs. Thus, the need for specific therapeutic agents capable of eliciting desired prophylactic immune responses against viral pathogens is met for influenza virus by this invention. Of particular importance in this therapeutic approach is the ability to induce T-cell immune responses which can prevent infections even of virus strains which are heterologous to the strain from which the antigen gene was obtained. Therefore, this invention provides DNA constructs encoding viral proteins of the human influenza virus nucleoprotein (NP), hemagglutinin (HA), neuraminidase (NM), matrix (M), nonstructural (NS), polymerase (PB1 and PB2= basic polymerases 1 and 2; PA= acidic polymerase) or any of the other influenza genes which encode products which generate specific CTLs.

The influenza virus has a ribonucleic acid (RNA) genome, consisting of multiple RNA segments. Each RNA encodes at least one gene product. The NP gene product binds to RNA and translocates viral RNA into the nucleus of the infected cell. The sequence is conserved, with only about 7% divergence in the amino acid sequence

having arisen over a period of 50 years. The P gene products (PB1, PB2, PA) are responsible for synthesizing new viral RNAs. These genes are even more highly conserved than the NP gene. HA is the major viral envelope gene product. It is less highly conserved than NP It binds a cellular receptor and is therefore instrumental in the initiation of new influenza infections. The major neutralizing antibody response is directed against this gene product. A substantial cytotoxic T lymphocyte response is also directed against this protein. Current vaccines against human influenza virus incorporate three strains of influenza virus or their HA proteins. However, due to the variability in the protein sequence of HA in different strains, the vaccine must constantly be tailored to the strains which are current in causing pathology. However, HA does have some conserved elements for the generation of CTLs, if properly presented. The NS1 and NS2 gene products have incompletely characterized biological functions, but may be significant in production of protective CTL responses. Finally, the M1 and M2 gene products, which are slightly more conserved than in HA, induce a major CTL response. The M1 protein is a very abundant viral gene product.

The protective efficacy of DNA vaccination against subsequent viral challenge is demonstrated by immunization with non-replicating plasmid DNA encoding one or more of the above mentioned viral proteins. This is advantageous since no infectious agent is involved, no assembly of virus particles is required, and determinant selection is permitted. Furthermore, because the sequence of nucleoprotein and several of the other viral gene products is conserved among various strains of influenza, protection against subsequent challenge by a virulent strain of influenza virus that is homologous to or heterologous to the strain from which the cloned gene is obtained is enabled.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1. Detection of NP plasmid DNA in muscle by PCR. Mice were injected three times, at three week intervals, with RSV-NP DNA or blank vector (100 μg/leg) into both quadriceps muscles of BALB/c mice, followed by influenza infection. The muscles were removed 4 weeks after the final injection and immediately frozen in liquid nitrogen. They were then pulverized in lysis buffer (25mM Tris-H_3PO_4 pH8, 2mM trans-1:2-diaminocyclohexan-tetra-acetic acid (CDTA), 2mM DTT, 10% glycerol, 1% Triton X-100) in a MIKRO-DISMEMBRATOR™ (B. Braun Instruments), and high molecular weight DNA was extracted by phenol/chloroform and ethanol precipitation. A 40 cycle PCR reaction (PCR was performed as per instructions in Perkin Elmer Cetus GENEAMP™ kit) was performed to detect the presence of NP plasmid DNA in muscle. A 772 base-pair PCR product (see arrowhead), which spans from the CMV promoter through most of the 5' portion of the inserted NP gene was generated from an 18 base long sense oligonucleotide which primed in the promoter region, (GTGTGCACCTCAAGCTGG, SEQ. ID:1:) and a 23 base long oligonucleotide antisense primer in the of the 5' portion of the inserted NP sequence (CCCTTTGAGAATGTTGCACATTC, SEQ. ID:2:). The 772 bp product is seen on an ethidium bromide-stained agarose gel in selected NP DNA-injected muscle samples but not in the blank vector control (600L). Labeling above each lane indicates mouse identification number and right or left leg.

Fig. 2. Production of NP antibodies in mice injected with NP DNA. Mice were injected with 100 μg V1-NP DNA in each leg at 0, 3 and 6 weeks, and blood was drawn on 0, 2, 5 and 8 weeks. The presence of anti-NP IgG in the serum was assayed by an ELISA (J. J. Donnelly *et al.*, J. Immunol. **145**, 3071 (1990)), with NP purified from insect cells that had been transfected with a baculovirus expression vector. The results are plotted as mean log10 ELISA titer ± SEM (n=10) against

time after the first injection of NP DNA. Mice immunized with blank vector generated no detectable NP antibodies.

Fig. 3. Percent specific lysis, determined in a 4-hour ^{51}Cr release assay, for CTLs obtained from mice immunized with DNA. Mice were immunized with 400 µg V1-NP DNA (solid circles) or blank vector (solid squares) and sacrificed 3-4 weeks later. Negative control CTL were obtained from a naive mouse (open triangles) and positive controls from a mouse that had recovered from infection with A/HK/68 four weeks previously (solid triangles). Graphs depict data from representative individual mice. At least eight individuals were studied for each set of conditions. Panel A: Spleen cells restimulated with NP147-155-pulsed autologous spleen cells and assayed against NP147-155-pulsed P815 cells. Panel B: Spleen cells restimulated with NP147-155-pulsed autologous spleen cells and assayed against P815 targets infected with influenza A/Victoria/73 (H3N2) for 6 hours before addition of CTL. Panel C: Spleen cells restimulated with Con A and IL-2 without additional antigen and assayed against P815 cells pulsed with NP147-155. Panel D: Mice were injected with 200 µg per injection of V1-NP DNA or blank vector three times at three week intervals. Spleens were harvested 4 weeks after the last immunization, spleen cells were cultured with IL-2 and Con A for 7 days, and CTL were assayed against P815 target cells infected with A/Victoria/73.

Fig. 4. Mass loss (in grams) and recovery in DNA-immunized mice after unanesthetized intranasal challenge with 10^4 TCID50 of A/HK/68. Mice were immunized three times at 3-week intervals with V1-NP DNA or blank vector, or were not injected, and were challenged 3 weeks after the last immunization. Weights for groups of 10 mice were determined at the time of challenge and daily from day 4 for NP DNA-injected mice (solid circles), blank vector controls (open triangles), and uninjected controls (open circles). Shown are mean weights ± SEM. NP DNA-injected mice displayed significantly less weight loss on day 8 through 13 than blank vector-injected ($p \leq 0.005$) and uninjected mice

(p≤0.01), as analyzed by the t-test. No significant difference was noted between the two controls (p=0.8 by the t-test).

Fig. 5. Survival of DNA immunized mice after intranasal challenge (under anesthesia) with $10^{2.5}$ $TCID_{50}$ of A/HK/68. Mice immunized three times at three week intervals with V1-NP DNA (closed circles) or blank vector (open circles) and uninjected controls (open triangles) were challenged three weeks after the final immunization. Percent survival is shown for groups of 9 or 10 mice. Survival of NP DNA-injected mice was significantly greater than controls (p=0.0004 by Chi-square analysis), while no significant difference was seen between blank vector-injected and uninjected mice (p=0.17 by Chi-square analysis).

Fig. 6. Sequence of the expression vector V1J, SEQ.ID:10:.

Fig. 7. Sequence of the expression vector V1Jneo, SEQ. ID:18:.

Fig. 8. Sequence of the CMVintA-BGH promoter-terminator sequence, SEQ. ID:11.

Fig. 9. Monkey anti-NP antibody

Fig. 10. Ferret hemagglutination inhibition, with the dotted line indicating the minimal protective antibody titer, and the average value being denoted with a circle having a line through it.

Fig. 11. IgG Anti-NP antibody in ferrets after DNA immunization.

Fig. 12. Influenza virus shedding in ferrets with and without DNA immunization.

Fig. 13. Diagram of pRSV-PR-NP and VI-NP vectors. X denotes the inserted coding region.

Fig. 14. Schematic of influenza proteins and strains.

Fig. 15. Schematic of injected DNA processing inside a cell.

Fig. 16. Resistence of ferrets to influenza A/RP/8/34 induced by immunization with HA and internal protein genes.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides nucleic acid pharmaceuticals which, when directly introduced into an animal, including vertebrates, mammals and humans, induce the expression of encoded proteins within the animal. Where the protein is one which does not normally occur in that animal except in pathological conditions, such as proteins associated with influenza virus, for example but not limited to the influenza nucleoprotein, neuraminidase, hemagglutinin, polymerase, matrix or nonstructural proteins, the animals' immune system is activated to launch a protective response. Because these exogenous proteins are produced by the animals' own tissues, the expressed proteins are processed and presented by the major histocompatibility complex, MHC. This recognition is analogous to that which occurs upon actual infection with the related organism. The result, as shown in this disclosure, is induction of immune responses which protect against virulent infection.

This invention provides nucleic acids which, when introduced into animal tissues in vivo, by injection, inhalation, or impression by an analogous mechanism (see BACKGROUND OF THE INVENTION above), the expression of the human influenza virus gene product occurs. Thus, for example, injection of DNA constructs of this invention into the muscle of mice, induces expression of the encoded gene products. Likewise, in ferrets and rhesus monkeys. Upon subsequent challenge with virulent influenza virus, using doses which uniformly kill control animals, animals injected with the nucleic acid therapeutic exhibit much reduced morbidity and mortality. Thus, this invention discloses a vaccine useful in humans to prevent influenza virus infections.

In one embodiment of the invention, the human influenza virus nucleoprotein, NP, sequence, obtained from the A/PR/8/34 strain, is cloned into an expression vector. The vector contains a promoter for RNA polymerase transcription, and a transcriptional terminator at the end of the NP coding sequence. In one preferred embodiment, the promoter is the Rous sarcoma virus (RSV) long terminal repeat (LTR) which is a strong transcriptional promoter. A more preferred promoter is the cytomegalovirus promoter with the intron A sequence (CMV-intA). A preferred transcriptional terminator is the bovine growth hormone terminator. The combination of CMVintA-BGH terminator is particularly preferred. In addition, to assist in preparation of the pharmaceutical, an antibiotic resistance marker is also preferably included in the expression vector. Ampicillin resistence genes, neomycin resistance genes or any other pharmaceutically acceptable antibiotic resistance marker may be used. In a preferred embodiment of this invention, the antibiotic resistence gene encodes a gene product for neomycin resistence. Further, to aid in the high level production of the pharmaceutical by fermentation in prokaryotic organisms, it is advantageous for the vector to contain an origin of replication and be of high copy number. Any of a number of commercially available prokaryotic cloning vectors provide these benefits. In a preferred embodiment of this invention, these functionalities are provided by the commercially available vectors known as pUC. It is desirable to remove non-essential DNA sequences. Thus, the lacZ and lacI coding sequences of pUC are removed in one embodiment of the invention.

In one embodiment, the expression vector pnRSV is used, wherein the rous sarcoma virus (RSV) long terminal repeat (LTR) is used as the promoter. In another embodiment, V1, a mutated pBR322 vector into which the CMV promoter and the BGH transcriptional terminator were cloned is used. The V1-NP construct was used to immunize mice and induce CTLs which protect against heterologous challenge. In a particularly preferred embodiment of this invention, the elements of V1 have been been combined to produce an expression

vector named V1J. Into V1J is cloned an influenza virus gene, such as an A/PR/8/34 NP, PB1, NS1, HA, PB2, or M1 gene. In yet another emobodiment, the ampicillin resistance gene is removed from V1J and replaced with a neomycin resistance gene, to generate v1J-neo, into which any of a number of different influenza virus genes have been cloned for use according to this invention.

While one embodiment of this invention incorporates the influenza NP gene from the A/PR/8/34 strain, more preferred embodiments incorporate an NP gene, an HA gene, an NM gene, a PB gene, a M gene, or an NS gene from more recent influenza virus isolates. This is accomplished by preparing cDNA copies of the viral genes and then subcloning the individual genes. Sequences for many genes of many influenza virus strains are now publicly available on GENEBANK (about 509 such sequences for influenza A genes). Thus, any of these genes, cloned from the recent Texas, Beijing or Panama isolates of the virus, which are strains recommended by the Center for Disease Control as being desirable in anti-influenza vaccines, are preferred in this invention (see FLU-IMMUNE® influenza virus vaccine of Lederle, Physicians Desk Reference, 1993, p1232, a trivalent purified influenza surface antigen vaccine containing the hemagglutinin protein from A/Texas/36/91, H1N1; A/Beijing/353/89, H3N2; and B/Panama/45/90). To keep the terminology consistent, the following convention is followed herein for describing DNA constructs: "Vector name-flu strain-gene". Thus, a construct wherein the NP gene of the A/PR/8/34 strain is cloned into the expression vector V1Jneo, the name it is given herein is: "V1Jneo-PR-NP". Naturally, as the etiologic strain of the virus changes, the precise gene which is optimal for incorporation in the pharmaceutical may change. However, as is demonstrated below, because cytotoxic lymphocyte responses are induced which are capable of protecting against heterologous strains, the strain variability is less critical in the novel vaccines of this invention, as compared with the whole virus or subunit polypeptide based vaccines. In addition, because the pharmaceutical is easily manipulated to insert a

new gene, this is an adjustment which is easily made by the standard techniques of molecular biology.

Because the sequence of nucleoprotein is conserved among various strains of influenza, protection was achieved against subsequent challenge by a virulent strain of influenza A that was heterologous to the strain from which the gene for nucleoprotein was cloned. Comparisons of NP from numerous strains of influenza A have shown no significant differences in secondary structure [M. Gammelin *et al*., Virol. **170**, 71, 1989] and very few changes in amino acid sequence [O. T. Gorman *et al*., J. Virol. **65**, 3704, 1991]. Over an approximately 50 year period, NP in human strains evolved at a rate of only 0.66 amino acid changes per year. Moreover, our results which show that A/HK/68-specific CTLs recognize target cells pulsed with the synthetic peptide NP(147-155) derived from the sequence of A/PR8/34 NP indicate that this H-2K^d-restricted CTL epitope has remained functionally intact over a 34 year span (see Figure 2). It should be noted also that where the gene encodes a viral surface antigen, such as hemagglutinin or even neuraminidase, a significant neutralizing humoral (antibody) immune response is generated in addition to the very important cytotoxic lymphocyte response.

The i.m. injection of a DNA expression vector encoding a conserved, internal protein of influenza A resulted in the generation of significant protective immunity against subsequent viral challenge. In particular, NP-specific antibodies and primary CTLs were produced. NP DNA immunization resulted in decreased viral lung titers, inhibition of weight loss, and increased survival, compared to controls. The protective immune response was not mediated by the NP-specific antibodies, as demonstrated by the lack of effect of NP antibodies alone (see Example 4) in combating a virus infection, and was thus likely due to NP-specific cellular immunity. Moreover, significant levels of primary CTLs directed against NP were generated. The protection was against a virulent strain of influenza A that was heterologous to the strain from which the DNA was cloned. Additionally, the challenge strain arose more than three decades after the A/PR/8/34 strain.

indicating that immune responses directed against conserved proteins can be effective despite the antigenic shift and drift of the variable envelope proteins. Because each of the influenza virus gene products exhibit some degree of conservation, and because CTLs may be generated in response to intracellular expression and MHC processing, it is predictable that other influenza virus genes will give rise to responses analogous to that achieved for NP. Thus, many of these genes have been cloned, as shown by the cloned and sequenced junctions in the expression vector (see below) such that these constructs are prophylactic agents in available form.

Therefore, this invention provides expression vectors encoding an influenza viral protein as an immunogen. The invention offers a means to induce cross-strain protective immunity without the need for self-replicating agents or adjuvants. In addition, immunization with DNA offers a number of other advantages. First, this approach to vaccination should be applicable to tumors as well as infectious agents, since the $CD8^+$ CTL response is important for both pathophysiological processes [K. Tanaka *et al.*, Annu. Rev. Immunol. **6**, 359 (1988)]. Therefore, eliciting an immune response against a protein crucial to the transformation process may be an effective means of cancer protection or immunotherapy. Second, the generation of high titer antibodies against expressed proteins after injection of viral protein (NP and hemagglutinin) and human growth hormone DNA, [see for example D.-c. Tang *et al.*, Nature **356**, 152, 1992], indicates this is a facile and highly effective means of making antibody-based vaccines, either separately or in combination with cytotoxic T-lymphocyte vaccines targeted towards conserved antigens.

The ease of producing and purifying DNA constructs compares favorably with traditional protein purification, facilitating the generation of combination vaccines. Thus, multiple constructs, for example encoding NP, HA, M1, PB1, NS1, or any other influenza virus gene may be prepared, mixed and co-administered. Finally, because protein expression is maintained following DNA injection [H. Lin *et al.*, Circulation **82**, 2217 (1990); R.N. Kitsis *et al.*, Proc. Natl. Acad. Sci.

(USA) **88**, 4138 (1991); E. Hansen *et al*., FEBS Lett. **290**, 73 (1991); S. Jiao *et al*., Hum. Gene Therapy 3, 21 (1992); J.A. Wolff *et al*., Human Mol. Genet. **1**, 363 (1992)], the persistence of B- and T-cell memory may be enhanced [D. Gray and P. Matzinger, J. Exp. Med. 174, 969 (1991); S. Oehen *et al*., *ibid*. 176, 1273 (1992)], thereby engendering long-lived humoral and cell-mediated immunity.

The standard techniques of molecular biology for preparing and purifying DNA constructs enable the preparation of the DNA therapeutics of this invention. While standard techniques of molecular biology are therefore sufficient for the production of the products of this invention, the specific constructs disclosed herein provide novel therapeutics which surprisingly produce cross-strain protection, a result heretofore unattainable with standard inactivated whole virus or subunit protein vaccines.

The amount of expressible DNA to be introduced to a vaccine recipient will depend on the strength of the transcriptional and translational promoters used in the DNA construct, and on the immunogenicity of the expressed gene product. In general, an immunologically or prophylactically effective dose of about 1 µg to 1 mg, and preferably about 10 µg to 300 µg is administered directly into muscle tissue. Subcutaneous injection, intradermal introduction, impression through the skin, and other modes of administration such as intraperitoneal, intravenous, or inhalation delivery are also contemplated. It is also contemplated that booster vaccinations are to be provided.

The DNA may be naked, that is, unassociated with any proteins, adjuvants or other agents which impact on the recipients immune sytem. In this case, it is desirable for the DNA to be in a physiologically acceptable solution, such as, but not limited to, sterile saline or sterile phosphate buffered saline. Alternatively, the DNA may be associated with liposomes, such as lecithin liposomes or other liposomes known in the art, as a DNA-liposome mixture, or the DNA may be associated with an adjuvant known in the art to boost immune responses, such as a protein or other carrier. Agents which assist in the

cellular uptake of DNA, such as, but not limited to, calcium ions, may also be used to advantage. These agents are generally referred to as pharmaceutically acceptable carriers.

Accordingly, one embodiment of this invention is a method for using influenza virus genes to induce immune responses <u>in vivo</u>, in a vertebrate such as a mammal, including a human, which comprises:

a) isolating the gene,

b) linking the gene to regulatory sequences such that the gene is operatively linked to control sequences which, when introduced into a living tissue direct the transcription initiation and subsequent translation of the gene,

c) introducing the gene into a living tissue, and

d) optionally, boosting with additional influenza gene.

A preferred embodiment of this invention is a method for protecting against heterologous strains of influenza virus. This is accomplished by administering an immunologically effective amount of a nucleic acid which encodes a conserved influenza virus epitope. For example, the entire influenza gene for nucleoprotein provides this function, and it is contemplated that coding sequences for the other influenza genes and portions thereof encoding conserved epitopes within these genes likewise provide cross-strain protection.

In another embodiment of this invention, the DNA vaccine encodes human influenza virus nucleoprotein, hemagglutinin, matrix, nonstructural, or polymerase gene product. Specific examples of this embodiment are provided below wherein the human influenza virus gene encodes the nucleoprotein, basic polymerase1, nonstructural protein1, hemagglutinin, matrix1, basic polymerase2 of human influenza virus isolate A/PR/8/34, the nucleoprotein of human influenza virus isolate A/Beijing/353/89, the hemagglutinin gene of human influenza virus isolate A/Texas/36/91, or the hemagglutinin gene of human influenza virus isolate B/Panama/46/90.

In specific embodiments of this invention, the DNA construct encodes an influenza virus gene, wherein the DNA construct is capable of being expressed upon introduction into animal tissues <u>in vivo</u>

and generating an immune response against the expressed product of the encoded influenza gene. Examples of such DNA constructs are:

a) pnRSV-PR-NP,
b) V1-PR-NP,
c) V1J-PR-NP, SEQ. ID:12:,
d) V1J-PR-PB1, SEQ. ID:13:,
e) V1J-PR-NS, SEQ. ID:14:,
f) V1J-PR-HA, SEQ. ID:15:,
g) V1J-PR-PB2, SEQ. ID:16:,
h) V1J-PR-M1, SEQ. ID:17:,
i) V1Jneo-BJ-NP, SEQ. ID:20: and SEQ. ID:21:,
j) V1Jneo-TX-NP, SEQ. ID:24 and SEQ. ID:25: and
k) V1Jneo-PA-HA, SEQ. ID:26: and SEQ. ID:27:.

The following examples are provided to further define the invention, without limiting the invention to the specifics of the examples.

EXAMPLE 1

PREPARATION OF DNA CONSTRUCTS ENCODING HUMAN INFLUENZA VIRUS PROTEINS:

i). pnRSV-PRNP: The A/PR/8/34 NP gene was isolated from pAPR-501 [J.F. Young *et al.*, in *The Origin of Pandemic Influenza Viruses*, W.G. Laver, Ed. (Elsevier Science Publishing Co., Inc., 1983)] as a 1565 base-pair EcoRI fragment, Klenow filled-in and cloned into the Klenow filled-in and phosphatase-treated XbaI site of pRSV-BL. pRSV-BL was constructed by first digesting the pBL-CAT3 [B. Luckow and G. Schutz, Nuc. Acids Res. **15**, 5490 (1987)] vector with Xho I and Nco I to remove the CAT coding sequence and Klenow filled-in and self-ligated. The RSV promoter fragment was isolated as an Nde I and Asp718 fragment from pRshgmx [V. Giguere et al., Nature **330**, 624 (1987)], Klenow filled-in and cloned into the HindIII site of the intermediate vector generated above (pBL-CAT lacking the CAT sequence).

ii) V1-NP: The expression vector V1 was constructed from pCMVIE-AKI-DHFR [Y. Whang *et al.*, J. Virol. **61**, 1796 (1987)]. The AKI and DHFR genes were removed by cutting the vector with EcoR I and self-ligating. This vector does not contain intron A in the CMV promoter, so it was added as a PCR fragment that had a deleted internal Sac I site [at 1855 as numbered in B.S. Chapman *et al.*, Nuc. Acids Res. **19**, 3979 (1991)]. The template used for the PCR reactions was pCMVintA-Lux, made by ligating the Hind III and Nhe I fragment from pCMV6a120 [see B.S. Chapman *et al.*, *ibid.*,] which includes hCMV-IE1 enhancer/promoter and intron A, into the Hind III and Xba I sites of pBL3 to generate pCMVIntBL. The 1881 base pair luciferase gene fragment (Hind III-Sma I Klenow filled-in) from RSV-Lux [J.R. de Wet *et al.*, Mol. Cell Biol. 7, 725, 1987] was cloned into the Sal I site of pCMVIntBL, which was Klenow filled-in and phosphatase treated.

The primers that spanned intron A are:
5' primer, SEQ. ID:5:

5'-CTATATAAGCAGAG CTCGTTTAG-3'.
The 3' primer, SEQ ID:6:
5'-GTTAGCAAAGATCTAAGGACGGTGA CTGCAG-3'.

The primers used to remove the Sac I site are:
sense primer, SEQ ID:7:
5'-GTATGTGTCTGAAAATGAGC<u>GT</u>GGAGATTGGGCTCGCAC-3'
and the antisense primer, SEQ ID:8:
5'-
GTGCGAGCCCAATCTCC<u>AC</u>GCTCATTTTCAGACACA TAC-3'.

The PCR fragment was cut with Sac I and Bgl II and inserted into the vector which had been cut with the same enzymes. The NP gene from Influenza A (A/PR/8/34) was cut out of pAPR501 [J.F. Young *et al.*, in *The Origin of Pandemic Influenza Viruses*, W.G. Laver, Ed. (Elsevier Science Publishing Co., Inc., 1983)] as a 1565 base-pair EcoR I fragment and blunted. It was inserted into V1 at the blunted Bgl II site, to make V1-NP. Plasmids were propagated in E. coli and purified by the alkaline lysis method [J. Sambrook, E.F. Fritsch, and T. Maniatis, in *Molecular Cloning, A Laboratory Manual*, second edition (Cold Spring Harbor Laboratory Press, 1989)]. CsCl banded DNA was ethanol precipitated and resuspended in 0.9% saline at 2mg/ml for injection.

EXAMPLE 2
ASSAY FOR HUMAN INFLUENZA VIRUS CYTOTOXIC T-LYMPHOCYTES:

Cytotoxic T lymphocytes were generated from mice that had been immunized with DNA or that had recovered from infection with A/HK/68. Control cultures were derived from mice that had been injected with control DNA and from uninjected mice. Single cell suspensions were prepared, red blood cells were removed by lysis with ammonium chloride, and spleen cells were cultured in RPMI 1640 supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.01 M HEPES (pH 7.5), and 2 mM l-glutamine. An equal number of autologous, irradiated stimulator cells,

pulsed for 60 min. with the H-2K^d-restricted peptide epitope NP147-155 (Thr Tyr Gln Arg Thr Arg Ala Leu Val, SEQ ID:9:) at 10 μM or infected with influenza A/PR8/34 (H1N1), and 10 U/ml recombinant human IL-2 (Cellular Products, Buffalo, NY) were added and cultures were maintained for 7 days at 37°C with 5% CO_2 and 100% relative humidity. In selected experiments, rhIL-2 (20 U/ml) and Con A (2 μg/ml) were added in place of autologous stimulator cells. Cytotoxic T cell effector activity was determined with P815 cells labeled for 3 hr with 60 μCi of ^{51}Cr per 10^6 cells, and pulsed as above with NP147-155, or infected with influenza A/Victoria/73 (H3N2). Control targets (labeled P815 cells without peptide or virus) were not lysed. Targets were plated at 1 x 10^4 cells/well in round-bottomed 96-well plates and incubated with effectors for 4 hours in triplicate. Supernatant (30 μl) was removed from each well and counted in a Betaplate scintillation counter (LKB-Wallac, Turku, Finland). Maximal counts, released by addition of 6M HCl, and spontaneous counts released without CTL were determined for each target preparation. Percent specific lysis was calculated as: [(experimental - spontaneous)/(maximal - spontaneous)] X 100.

EXAMPLE 3

PRODUCTION OF NP SPECIFIC CTLs AND ANTIBODIES IN VIVO:

BALB/c mice were injected in the quadriceps of both legs with plasmid cDNA encoding A/PR/8/34 nucleoprotein driven by either a Rous sarcoma virus or cytomegalovirus promoter.

Expression vectors used were:
i) pnRSV-PRNP, see Example 1;
ii) V1-NP, see Example 1.

Animals used were female BALB/c mice, obtained from Charles River Laboratories, Raleigh, NC. Mice were obtained at 4-5 weeks of age and were initially injected with DNA at 5-6 weeks of age. Unless otherwise noted, injections of DNA were administered into the quadriceps muscles of both legs, with each leg receiving 50 μl of sterile saline containing

100 µg of DNA. Mice received 1, 2 or 3 sets of inoculations at 3 week intervals. Negative control animals were uninjected or injected with the appropriate blank vector lacking the inserted NP gene.

The presence or absence of NP plasmid DNA in the muscles of selected animals was analyzed by PCR (Fig. 1). Plasmid DNA (either NP or luciferase DNA) was detected in 44 of 48 injected muscles tested. In mice injected with luciferase DNA, protein expression was demonstrated by luciferase activity recovered in muscle extracts according to methods known in the art [J.A. Wolff *et al.*, Science **247**, 1465 (1990); G. Ascadi *et al.*, Nature **352**, 815 (1991); H. Lin *et al.*, Circulation **82**, 2217 (1990); R.N. Kitsis *et al.*, Proc. Natl. Acad. Sci. (USA) **88**, 4138 (1991); E. Hansen *et al.*, FEBS Lett. **290**, 73 (1991); S. Jiao *et al.*, Hum. Gene Therapy 3, 21 (1992); J.A. Wolff *et al.*, Human Mol. Genet. **1**, 363 (1992)].

NP expression in muscles after injection of NP DNA was below the limit of detection for Western blot analysis (< 1 ng) but was indicated by the production of NP-specific antibodies (see Fig. 2). For analysis of NP-specific CTL generation, spleens were removed 1-4 weeks following immunization, and spleen cells were restimulated with recombinant human IL-2 plus autologous spleen cells that had been either infected with influenza A (A/PR/8/34) or pulsed with the H-2K^d-restricted nucleoprotein peptide epitope (NP residues 147-155, see O.K. Rötzscke *et al.*, Nature **348**, 252 (1990)). Spleen cells restimulated with virally-infected or with epitope-pulsed syngeneic cells were capable of killing nucleoprotein epitope-pulsed target cells (Fig. 3A). This indicates that i.m. injection of NP DNA generated the appropriate NP-derived peptide in association with MHC class I for induction of the specific CTL response. These CTLs were capable of recognizing and lysing virally infected target cells (Fig 3B), or target cells pulsed with the H-2K^d-restricted nucleoprotein peptide epitope and virally-infected target cells. This demonstrates their specificity as well as their ability to detect the epitope generated naturally in infected cells.

A more stringent measure of immunogenicity of the NP DNA vaccine was the evaluation of the primary CTL response. Spleen cells

taken from NP DNA-injected mice were activated by exposure to Con A and IL-2, but did not undergo in vitro restimulation with antigen-expressing cells prior to testing their ability to kill appropriate targets. Splenocytes from mice immunized with NP DNA, when activated with Con A and IL-2 in vitro without antigen-specific restimulation, lysed both epitope-pulsed and virally-infected target cells (Fig. 3C and D). This lytic activity of both the restimulated and activated spleen cells compares favorably with that of similarly treated splenocytes derived from mice that had been previously infected with influenza A/HK/68, a virulent mouse-adapted H3N2 strain that arose 34 years after A/PR/8/34 (H1N1). Thus, injection of NP DNA generated CTL that were specific for the nucleoprotein epitope and that were capable of identifying the naturally processed antigen.

Injection of mice with NP DNA resulted in the production of high titer anti-NP IgG antibodies (Fig. 2). Generation of high titer IgG antibodies in mice is thought to require CD4+ T cell help (P. Vieira and K. Rajewsky, Int. Immunol. 2, 487 (1990); J. J. Donnelly *et al.*, J. Immunol. **145**, 3071 (1990)). This shows that NP expressed from the plasmid in situ was processed for presentation by both MHC class I and class II.

EXAMPLE 4
PROTECTION OF MICE UPON CHALLENGE WITH VIRULENT HUMAN INFLUENZA VIRUS:

The role of NP antibodies in protective immunity to influenza is shown by two approaches: First, viral lung titers were determined in a passive-transfer experiment. Female BALB/c mice ≥ 10 weeks of age were injected intraperitoneally with 0.5 ml of pooled serum (diluted in 2.0 ml of PBS) from mice that had been injected 3 times with 200 μg of NP DNA. Control mice were injected with an equal volume of pooled normal mouse serum, or with pooled serum from mice that had recovered from infection with A/HK/68, also in 2.0 ml of PBS. The dose of A/HK/68 immune serum was adjusted such that the ELISA titer of anti-NP antibody was equal to that in the pooled serum from NP

DNA-injected mice. Mice were challenged unanesthetized in a blinded fashion with 10^4 TCID50 of A/HK/68 2 hours after serum injection, and a further injection of an equal amount of serum was given 3 days later. Mice were sacrificed 6 and 7 days after infection and viral lung titers in TCID50 per ml were determined as described by Moran [J. Immunol. **146**, 321, 1991].

Naive mice were infused with anti-NP antiserum, obtained from mice that were injected with NP DNA, and then challenged with A/HK/68 . Viral challenges were performed with a mouse-adapted strain of A/HK/68 and maintained subsequently by *in vivo* passage in mice (Dr. I. Mbawuike, personal communication). The viral seed stock used was a homogenate of lungs from infected mice and had an infectivity titer of 5 x 10^8 TCID50/ml on MDCK cells. For viral lung titer determinations and weight loss studies, viral challenges were performed in blinded fashion by intranasal instillation of 20 µl containing 10^4 TCID50 onto the nares of unanesthetized mice, which leads to progressive infection of the lungs with virus but is not lethal in BALB/c mice [Yetter, R.A. *et al.*, Infect. Immunity **29**, 654, 1980]. In survival experiments, mice were challenged by instillation of 20 µl containing $10^{2.5}$ TCID50 onto the nares under full anesthesia with ketamine and xylazine; infection of anesthetized mice with this dose causes a rapid lung infection which is lethal to 90-100% of nonimmunized mice [J.L. Schulman and E.D. Kilbourne, J. Exp. Med. **118**, 257, 1963; G.H. Scott and R.J. Sydiskis, Infect. Immunity **14**, 696, 1976; R.A. Yetter *et al.*, Infect. Immunity **29**, 654, 1980]. Viral lung titers were determined by serial titration on MDCK cells (obtained from ATCC, Rockville, MD) in 96-well plates as described by Moran *et al.*, [*ibid.*].

No reduction in viral lung titers was seen in mice that had received anti-NP antiserum (6.3 ± 0.2; mean ± SEM; n=4) as compared to control mice that had received normal serum (6.1 ± 0.3; mean ± SEM; n=4). As a positive control, serum was collected from mice that had been infected with A/HK/68 and passively transferred to four naive mice. After a challenge with A/HK/68, no viral infection was detectable

in their lungs, indicating that this serum against whole virus was completely protective for challenge with the homologous virus. Second, naive mice were immunized with purified NP (5 µg/leg, 3 times over a period of 6 weeks) by i.m. injection. These mice generated high titer NP-specific antibodies but failed to produce NP-specific CTLs and were not protected from a lethal dose of virus. Therefore, unlike the neutralizing effect of antibodies to whole virus, circulating anti-NP IgG did not confer protective immunity to the mice.

The in vivo protective efficacy of NP DNA injections was evaluated to determine whether a cell-mediated immune response was functionally significant. One direct measure of the effectiveness of the immune response was the ability of mice first immunized with NP DNA to clear a progressive, sublethal lung infection with a heterologous strain of influenza (A/HK/68: H3N2). Viral challenges were conducted as described above. Mice immunized with NP DNA had viral lung titers after challenge that were three orders of magnitude lower on day 7 (1.0 ± 1.0; mean ± SEM; n=4) than those of control mice that had not been immunized (4.1 ± 0.3; mean ± SEM; n=4), or that had been immunized with blank vector (4.5 ± 0.0; mean ± SEM; n=4). In fact, three of four immunized mice had undetectable levels of virus in their lungs, while none of the controls had cleared virus at this point. The substantial difference in the viral lung titers seen in this experiment and six others demonstrates that the immune response accelerated clearance of the virus. The lack of protective effect of the blank vector control confirms that DNA per se was not responsibie for the immune response. Moreover, because the challenge strain of virus, A/HK/68 (a virulent, mouse-adapted H3N2 strain), was heterologous to the strain A/PR8/34 (H1N1) from which the NP gene was cloned, the immunity was clearly heterotypic.

As a measure of virus-induced morbidity, the mass loss was monitored in mice that were infected sublethally with influenza A/HK/68 following immunization with NP DNA (Fig. 4). Uninjected mice or mice injected with the blank vector were used as controls. Mice immunized with NP DNA exhibited less weight loss and a more rapid

return to their pre-challenge weights following influenza A infection compared to control mice.

Intranasal infection of fully anesthetized mice with influenza A causes rapid widespread viral replication in the lung and death in 6-8 days if the infection is not controlled (R.A. Yetter *et al.*, Infect. Immunity 29, 654 (1980)). Survival of mice challenged by this method reflects their ability to limit the severity of an acute lung infection. The capacity of mice to survive challenge with two different strains of influenza, A/HK/68 (see Fig. 5) and A/PR/8/34, was studied. Mice previously immunized with NP DNA showed a 90% survival rate compared to 0% in blank vector injected and 20% in uninjected control animals (Fig. 5). In a total of 14 such studies, mice immunized with NP DNA showed at least a 50% greater survival rate than controls. Thus, the ability of the NP DNA-induced immune response to effectively accelerate recovery and decrease disease caused by a virus of a different strain arising 34 years later supports the rationale of targeting a conserved protein for the generation of a cytotoxic T-lymphocyte response.

EXAMPLE 5
ISOLATION OF GENES FROM INFLUENZA VIRUS ISOLATES:

Many of the older influenza virus strains are on deposit with the ATCC (the 1990 Catalogue of Animal Viruses & Antisera, Chlamydiae & Rickettsiae, 6th edition, lists 20 influenza A strains and 14 influenza B strains.

A. Viral Strains and Purification:

Influenza strains which comprise the current, 1992 flu season vaccine were obtained from Dr. Nancy J. Cox at the Division of Viral and Rickettsial Diseases, Centers of Disease Control, Atlanta, GA. These strains are: (1) A/Beijing/353/89 (H3N2); (2) A/Texas/36/91 (H1N1); and (3) B/Panama/45/90.

These viruses were grown by passage in 9- to 11-day-old embryonated chicken eggs (100-200 per viral preparation) and purified by a modification of the method described by Massicot et al. (Virology

101, 242-249 (1980)). In brief, virus suspensions were clarified by centrifugation at 8000 rpm (Sorvall RC5C centrifuge, GS-3 rotor) and then pelleted by centrifugation at 18,000 rpm for 2 h in a Beckman Type 19 rotor. The pelleted virus was resuspended in STE (0.1 M NaCl, 20 mM Tris, pH 7.4, 1 mM EDTA) and centrifuged at 4,000 rpm for 10 min (Hermle Z 360 K centrifuge) to remove aggregates. 2 ml of supernatant was layered onto a discontinuous sucrose gradient consisting of 2 ml of 60% sucrose overlayed with 7 ml of 30% sucrose buffered with STE and centrifuged at 36,000 rpm (SW-40 rotor, Beckman) for 90 minutes. Banded virus was collected at the interface, diluted 10-fold with STE, and pelleted at 30,000 rpm for 2 h (Beckman Ti45 rotor). The pelleted virus was then frozen at -70^{o}C.

B. Extraction of Viral RNA and cDNA Synthesis:

Viral RNA was purified from frozen virus by guanidinium isothiocyanate extraction using a commercially available kit (Stratagene, La Jolla, CA) employing the method of Chomczynski and Sacchi (Anal. Biochem. 162, 156-159 (1987)). Double-stranded cDNA was prepared from viral RNA using a commercially available cDNA synthesis kit (Pharmacia) as directed by the manufacturers with several modifications. The first strand of cDNA was primed using a synthetic oligodeoxyribonucleotide, 5'-AGCAAAAGCAGG-3', SEQ. ID:30:, which is complementary to a conserved sequence located at the 3'-terminus of the viral RNA. This sequence is common to all type A influenza viral RNAs. After synthesis of first and second strands of cDNA the reactions were extracted with phenol/chloroform and ethanol precipitated rather than continuing with the kit directions. These blunt-ended cDNA's were then directly ligated into V1Jneo vector which had been digested with the BglII restriction enzyme, blunt-ended with T4 DNA polymerase, and treated with calf intestinal alkaline phosphatase.

To screen for particular full-length viral genes we used synthetic oligodeoxyribonucleotides which were designed to complement the 3'-terminus of the end of the translational open reading frame of a given viral gene. Samples which appeared to represent full-length genes by

restriction mapping and size determination on agarose electrophoresis gels were verified by dideoxynucleotide sequencing of both junctions of the viral gene with V1Jneo. The sequence junctions for each gene cloned from these viruses is given below in Example 8.

Similar strategies were used for cloning cDNA's from each of the viruses named above except that for B/Panama/45/90, which does not have common sequences at each end of viral RNA, a mixture of oligodeoxyribonucleotides were used to prime first strand cDNA synthesis. These primers were:

(1) 5'-AGCAGAAGCGGAGC-3', SEQ. ID:31: for PB1 and PB2;
(2) 5'-AGCAGAAGCAGAGCA-3', SEQ. ID:19: for NS and HA;
(3) 5'-AGCAGAAGCACGCAC-3', SEQ. ID:22: for M; and
(4) 5'-AGCAGAAGCACAGCA-3', SEQ. ID:23: for NP.

EXAMPLE 6
V1J EXPRESSION VECTOR, SEQ. ID:10:

Our purpose in creating V1J was to remove the promoter and transcription termination elements from our vector, V1, in order to place them within a more defined context, create a more compact vector, and to improve plasmid purification yields.

V1J is derived from vectors V1, (see Example 1) and pUC18, a commercially available plasmid. V1 was digested with SspI and EcoRI restriction enzymes producing two fragments of DNA. The smaller of these fragments, containing the CMVintA promoter and Bovine Growth Hormone (BGH) transcription termination elements which control the expression of heterologous genes (SEQ ID:11:), was purified from an agarose electrophoresis gel. The ends of this DNA fragment were then "blunted" using the T4 DNA polymerase enzyme in order to facilitate its ligation to another "blunt-ended" DNA fragment.

pUC18 was chosen to provide the "backbone" of the expression vector. It is known to produce high yields of plasmid, is well-characterized by sequence and function, and is of minimum size. We removed the entire *lac* operon from this vector, which was unnecessary for our purposes and may be detrimental to plasmid yields and heterologous gene expression, by partial digestion with the HaeII restriction enzyme. The remaining plasmid was purified from an

agarose electrophoresis gel, blunt-ended with the T4 DNA polymerase, treated with calf intestinal alkaline phosphatase, and ligated to the CMVintA/BGH element described above. Plasmids exhibiting either of two possible orientations of the promoter elements within the pUC backbone were obtained. One of these plasmids gave much higher yields of DNA in E. coli and was designated V1J (SEQ. ID:10:). This vector's structure was verified by sequence analysis of the junction regions and was subsequently demonstrated to give comparable or higher expression of heterologous genes compared with V1.

EXAMPLE 7

INFLUENZA VIRUS GENE CONSTRUCTS IN EXPRESSION VECTOR V1J:

Many of the genes from the A/PR/8/34 strain of influenza virus were cloned into the expression vector V1J, which, as noted in Example 4, gives rise to expression at levels as high or higher than in the V1 vector. The PR8 gene sequences are known and available in the GENEBANK database. For each of the genes cloned below, the size of the fragment cloned was checked by sizing gel, and the GENEBANK accession number against which partial sequence was compared are provided. For a method of obtaining these genes from virus strains, for example from virus obtained from the ATCC (A/PR/8/34 is ATCC VR-95; many other strains are also on deposit with the ATCC), see Example 5.

A. Subcloning the PR8 Genes into V1J:

1. NP gene

The NP gene was subcloned from pAPR501 (J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138). It was excised by cutting pAPR501 with EcoRI, the fragment gel purified, and blunted with T4 DNA Polymerase. The blunted fragment was inserted into V1J cut with Bgl II and also blunted with T4 DNA Polymerase. The cloned fragment was 1.6 kilobases long.

2. NS

The NS gene was subcloned from pAPR801 (J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138). It was excised by cutting pAPR801 with EcoRI, the fragment gel purified, and blunted with T4 DNA Polymerase. The blunted fragment was inserted into V1J cut with Bgl II and also blunted with T4 DNA Polymerase. The cloned fragment was 0.9 kilobases long (the complete NS coding region including NS1 and NS2).

3. HA

The HA gene was subcloned from pJZ102 (J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138). It was excised by cutting pJZ102 with Hind III, the fragment gel purified, and blunted with T4 DNA Polymerase. The blunted fragment was inserted into V1J cut with Bgl II and also blunted with T4 DNA Polymerase. The cloned fragment was 1.75 kilobases long.

4. PB1

The PB1 gene was subcloned from pGem1-PB1 (The 5' and 3' junctions of the genes with the vector were sequenced to verify their identity. See J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138). It was excised by cutting pGem-PB1 with Hind III, the fragment gel purified, and blunted with T4 DNA Polymerase. The blunted fragment was inserted into V1J cut with Bgl II and also blunted with T4 DNA Polymerase. The cloned fragment was 2.3 kilobases long.

5. PB2

The PB2 gene was subcloned from pGem1-PB2 (The 5' and 3' junctions of the genes with the vector were sequenced to verify their identity. See J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic

Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138). It was excised by cutting pGem-PB2 with BamH I, and gel purifying the fragment. The sticky-ended fragment was inserted into V1J cut with Bgl II. The cloned fragment was 2.3 kilobases long.

6. M1

The M1 gene was generated by PCR from the plasmid p8901 MITE. The M sequence in this plasmid was generated by PCR from pAPR701 (J.F. Young, U. Desselberber, P. Graves, P. Palese, A. Shatzman, and M. Rosenberg (1983), in The Origins of Pandemic Influenza Viruses, ed. W.G. Laver, (Elsevier, Amsterdam) pp.129-138.), using the oligomer 5'-GGT ACA AGA TCT ACC ATG CTT CTA ACC GAG GTC-3', SEQ. ID:3:, for the "sense" primer and the oligomer 5'-CCA CAT AGA TCT TCA CTT GAA CCG TTG CAT CTG CAC-3', SEQ. ID:4:, for the "anti-sense" primer. The PCR fragment was gel purified, cut with Bgl II and ligated into V1J cut with Bgl II. The cloned fragment was 0.7 kilobases long. The amino terminus of the encoded M1 is encoded in the "sense" primer shown above as the "ATG" codon, while the M1 translation stop codon is encoded by the reverse of the "TCA" codon, which in the sense direction is the stop codon "TGA".

B. Influenza Gene-V1J Expression Constructs:

In each case, the junction sequences from the 5' promoter region (CMVintA) into the cloned gene is shown. The sequences were generated by sequencing off the primer:
CMVinta primer 5'- CTA ACA GAC TGT TCC TTT CCA TG- 3', SEQ. ID:28:, which generates the sequence of the coding sequence. The position at which the junction occurs is demarcated by a "/", which does not represent any discontinuity in the sequence. The method for preparing these constructs is summarized after all of the sequences below. Each sequence provided represents a complete, available, expressible DNA construct for the designated influenza gene.

Each construct was transiently transfected into RD cells, (ATCC CCL136), a human rhabdomyosarcoma cell line in culture. Forty eight hours after transfection, the cells were harvested, lysed, and western blots were run (except for the V1J-PR-HA construct which was tested in mice and gave anti-HA specific antibody before a western blot was run, thus obviating the need to run a western blot as expression was observed

in vivo). Antibody specific for the PB1, PB2 and NS proteins was provided by Stephen Inglis of the University of Cambridge, who used purified proteins expressed as ß-galactosidase fusion proteins to generate polyclonal antisera. Anti-NP polyclonal antiserum was generated by immunization of rabbits with whole A/PR/8/34 virus. Anti-M1 antibody is commercially available from Biodesign as a goat, anti-fluA antiserum, catalog number B65245G. In each case, a protein of the predicted size was observed, confirming expression in vitro of the encoded influenza protein.

The nomenclature for these constructs follows the convention: "Vector name-flu strain-gene". In every case, the sequence was checked against known sequences from GENEBANK for the cloned and sequenced A/PR/8/34 gene sequence. The biological efficacy of each of these constructs is demonstrated as in Examples 2, 3, and 4 above:

SEQUENCE ACROSS THE 5' JUNCTIONS OF CMVINTA AND FLU GENES FROM A/PR/8/34:

1. V1J-PR-NP, SEQ. ID:12:, GENEBANK ACCESSION #:M38279
5' GTC ACC GTC CTT AGA TC/A ATT CCA GCA AAA GCA GGG
CMVintA NP....

TAG ATA ATC ACT CAC TGA GTG ACA TCA AAA TCA TG

2. V1J-PR-PB1, SEQ. ID:13:, GENEBANK ACCESSION #J02151
5' ACC GTC CTT AGA TC/A GCT TGG CAA AAG CAG GCA AAC
CMVintA PB1....

CAT TTG AAT GGA TGT CAA TCC GAC CTT ACT TTT CTT
AAA AGT GCC AGC ACA AAA TGC TAT AAG CAC AAC TTT
CCC TTA TAC

3. V1J-PR-NS, SEQ. ID:14:, GENEBANK ACCESSION #J02150
5' GTC ACC GTC CTT AGA TC/A ATT CCA GCA AAA GCA GGG
CMVintA NS....

TGA CAA AAA CAT AAT GGA TCC AAA CAC TGT GTC AAG
CTT TCA GGT AGA TTG CTT TCT TTG GCA TGT CCG CAA
ACG AGT TGC AGA CCA AGA ACT AGG TGA T...

4. VIJ-PR-HA, SEQ. ID:15:, GENEBANK ACCESSION #J02143
5' TCT GCA GTC ACC GTC CTT AGA TC/ A GCT TGG AGC AAA
CMVintA HA...

AGCAGG GGA AAA TAA AAA CAA CCA AAA TGA AGG CAA ACC TAC TGG TCC TGT TAA GTG CAC TTG CAG CTG CAG ATG CAG ACA CAA TAT GTA TAG GCT ACC ATG CGA ACA ATT CAA CC...

5. VIJ-PR-PB2, SEQ. ID:16:, GENEBANK ACCESSION #J02153
5'TTT TCT GCA GTC ACC GTC CTT AGA TC/ C CGA ATT CCA
CMVintA PB2....

GCA AAA GCA GGT CAA TTA TAT TCA ATA TGG AAA GAA TAA AAG AAC TAA GAA ATC TAA TGT CGC AGT CTG CCA CCC CGG AGA TAC TCA CAA AAA CCA CCG TGG ACC ATA TGG CCA TAA TCA AGA AGT...

6. VIJ-PR-M1, SEQ. ID:17:, GENEBANK ACCESSION #J02145
5' GTC ACC GTC CTT AGA TCT/ ACC ATG AGT CTT CTA ACC
CMVINTA M1.....

GAG GTC GAA ACG TAC GTA CTC TCT ATC ATC CCG TCA GGC CCC CTC AAA GCC GAG ATC GCA CAG AGA CTT GAA GAG TTG ACG GAA GA...

How Fragments were joined:

1. VIJ-PR-NP: Blunted BglII (vector) to blunted EcoRI (NP)
2. VIJ-PR-PB1: Blunted BglII (vector) to blunted HinDIII (PB1)
3. VIJ-PR-NS: Blunted BglII (vector) to blunted EcoRI (NS1)
4. VIJ-PR-HA: Blunted BglII (vector) to blunted HinDIII (HA)
5. VIJ-PR-PB2: Sticky BglII (vector) to sticky BamHI (PB2)

6. V1J-PR-M1: Sticky BglII (vector) to sticky BglII (M1) M1 was obtained by PCR, using p8901-M1TE as template and Primers that add a BglII site at both ends and start 3 bases befor the ATG and end right after the termination codon for M1 (TGA).

EXAMPLE 8
V1Jneo EXPRESSION VECTOR, SEQ. ID:18:

It was necessary to remove the amp^r gene used for antibiotic selection of bacteria harboring V1J because ampicillin may not be used in large-scale fermenters. The amp^r gene from the pUC backbone of V1J was removed by digestion with SspI and Eam1105I restriction enzymes. The remaining plasmid was purified by agarose gel electrophoresis, blunt-ended with T4 DNA polymerase, and then treated with calf intestinal alkaline phosphatase. The commercially available kan^r gene, derived from transposon 903 and contained within the pUC4K plasmid, was excised using the PstI restriction enzyme, purified by agarose gel electrophoresis, and blunt-ended with T4 DNA polymerase. This fragment was ligated with the V1J backbone and plasmids with the kan^r gene in either orientation were derived which were designated as V1Jneo #'s 1 and 3. Each of these plasmids was confirmed by restriction enzyme digestion analysis, DNA sequencing of the junction regions, and was shown to produce similar quantities of plasmid as V1J. Expression of heterologous gene products was also comparable to V1J for these V1Jneo vectors. We arbitrarily selected V1Jneo#3, referred to as V1Jneo hereafter (SEQ. ID:18:), which contains the kan^r gene in the same orientation as the amp^r gene in V1J as the expression construct.

Genes from each of the strains A/Beijing/353/89, A/Texas/36/91, and B/Panama/46/90 were cloned into the vector V1Jneo. In each case, the junction sequences from the 5' promoter region (CMVintA) into the cloned gene was sequenced using the primer:
CMVinta primer 5'- CTA ACA GAC TGT TCC TTT CCA TG- 3', SEQ. ID:28:, which generates the sequence of the coding sequence. This is contiguous with the terminator/coding sequence, the junction of which is also shown. This sequence was generated using the primer: BGH primer 5'- GGA GTG GCA CCT TCC AGG -3', SEQ. ID:29:, which generates the sequence of the non-coding strand. In every case, the sequence was checked against known sequences from GENEBANK for cloned and sequenced genes from these or other influenza isolates.

The position at which the junction occurs is demarcated by a "/", which does not represent any discontinuity in the sequence. In the case of the V1Jneo-TX-HA junction, the sequencing gel was compressed and the initial sequence was difficult to read. Therefore, the first 8 bases at that junction are shown as "N". The first "ATG" encountered in each sequence is the translation initiation codon for the respective cloned gene. Each sequence provided represents a complete, available, expressible DNA construct for the designated influenza gene. The nomenclature follows the convention: "Vector name-flu strain-gene". The biological efficacy of each of these constructs is shown in the same manner as in Examples 2, 3, and 4 above:

SEQUENCE ACROSS THE 5' JUNCTIONS OF CMVintA AND THE FLU GENES AND ACROSS THE 3' JUNCTIONS OF THE FLU GENES AND THE BGH TERMINATOR EXPRESSION CONSTRUCTS, USING DIFFERENT INFLUENZA STRAINS AND PROTEINS:

I. A/BEIJING/353/89

A. V1Jneo-BJ-NP:

PROMOTER, SEQ. ID:20:

5' TCA CCG TCC TTA GAT C/ AA GCA GGG TTA ATA ATC
CMVintA NP....
ACT CAC TGA GTG ACA TCA AAA TC ATG GCG TCC CAA GGC
ACC AAA CGG TCT TAT GAA CAG ATG GAA ACT GAT GGG
GAA CGC CAG ATT

TERMINATOR, SEQ. ID:21:

5' GAG GGG CAA ACA ACA GAT GGC TGG CAA CTA GAA GGC
ACA GCA GAT / ATT TTT TCC TTA ATT GTC GTA C...
BGH NP....

II. A/TEXAS/36/91

A. V1Jneo-TX-HA

PROMOTER, SEQ. ID:24.

5' CCT TAG ATC / NNN NNN NNA CAA CCA AAA TGA
CMVINTA HA....

AAG CAA AAC TAC TAG TCC...

TERMINATOR, SEQ. ID:25:

5' GCA GAT C/ CT TAT ATT TCT GAA ATT CTG GTC...
BGH HA....

TCA GAT. .

III. B/PANAMA/46/90

A. V1Jneo-PA-HA

PROMOTER, SEQ. ID:26: (The first 1080 bases of this sequence is available on GENEBANK as accession number M65171; the sequence obtained below is identical with the known sequence; the 3' sequence, SEQ. ID:27: below) has not been previously reported)

5'ACC GTC CTT AGA TC/ C AGA AGC AGA GCA TTT TCT AAT

CMVintA HA....

ATC CAC AAA ATG AAG GCA ATA ATT GTA CTA CTC ATG
GTA GTA ACA TCC AAC GCA GAT CGA ATC TGC...

TERMINATOR, SEQ. ID:27:

5' GGC ACA GCA GAT C/ TT TCA ATA ACG TTT CTT TGT
BGH HA....

AAT GGT AAC...

EXAMPLE 9

Intradermal Injections of Influenza Genes:

The protocol for intradermal introduction of genes was the same as for intramuscular introduction: Three injections of 200µg each, three weeks apart, of V1-PR-NP. The spleens were harvested for the in vitro assay 55 days after the third injection, and restimulated with the nonapeptide nucleoprotein epitope 147-155, SEQ. ID:9:. Target cells (P815 cells, mouse mastocytoma, syngeneic with BALB/c mice H-2^d) were infected with the heterologous virus A/Victoria/73, and specific lysis using the spleen cells as the effector at effector:target ratios ranging between 5:1 and 40:1. Negative controls were carried out by measuring lysis of target cells which were not infected with influenza virus. Positive controls were carried out by measuring lysis of influenza virus infected target cells by spleen cells obtained from a mouse which was injected three times with 130 µg of V1-PR-NP and which survived a live influenza virus infection by strain A/HK/68.

Results: Specific lysis was achieved using the spleen cells from intradermally injected mice at all effector:target ratios. No specific lysis was seen when spleen cells obtained from uninjected mice, or mice injected with the vector V1 without the inserted PR-NP gene, were used as the effector cells. In addition, the specific lysis achieved using the intradermal delivery was comparable at all effector:target ratios to the results obtained using intramuscular delivery. Influenza virus lung titers were also measured in mice injected intradermally or intramusculary. The results, using 5 mice per group, 3 x 200 µg per dose three weeks apart, and challenge 3 weeks post last dose, were as follows:

Vaccine	Mode of Delivery	Mouse Lung Titer*	
		Day 5	Day 7
V1-PR-NP	Intradermal	5.2 ± 0.2	4.1 ± 1**
V1	Intradermal	5.9 ± 1	6.6 ± 0.3
V1-PR-NP	Intramuscular	4.6 ± 0.4	4.5 ± 1.1**
None	---------------	6.2 ± 0.3	5.9 ± 0.3

* Mean log titer ± SEM.

** One mouse had no virus at all.

Finally, percent survival of mice was tested out to twenty eight days. By day twenty eight, of the mice receiving V1-NP-PR, 89% of the i.m. recipients and 50% of the i.d. recipients survived. None of the V1 vector and only 30% of the untreated mice survived. This experiment demonstrates that DNA encoding nucleoprotein from the A/PR/8/34 strain was able to induce CTL's that recognized the nucleoprotein from the hetereologous strain A/Victoria/73 and a protective immune response against the heterologous strain A/HK/68.

EXAMPLE 10

Polynucleotide vaccination in primates

1. Antibody to NP in Rhesus monkeys: Rhesus monkeys (006 NP, 009 NP or control 101; 021) were injected with 1 mg/site of RSV-NP i.m. in 3 sites on day 1. Injections of 1 mg each of RSV-LUX and CMV-int-LUX, constructs for the reporter gene firefly luciferase expression, were given at the same time into separate sites. Animals were re-injected on day 15 with the same amounts of DNA as before and also with 1 mg of pD5-CAT, a construct for the reporter gene chloramphenical acetyl transferase expression, in 1 site each. Muscle sites containing reporter genes were biopsied and assayed for reporter gene activity. Serum was collected 3, 5, 9, 11, 13, and 15 weeks after the first injection. The first positive sample for anti-NP antibody was collected at week 11 and positive samples were also collected on weeks 13 and 15. Anti-NP antibody was determined by ELISA. The results are shown in Figure 9.

2. Hemagglutination inhibiting (HI) antibody in rhesus monkeys: Monkeys were injected i.m. with V1J-PR-HA on day 1. Two animals each received 1 mg, 100 µg, or 10 µg DNA in each quadriceps muscle. Each injection was administered in a volume of 0.5 ml. Animals were bled prior to injection on day 1. All animals were reinjected with DNA on day 15, and blood was collected at 2-4 week intervals thereafter. Hemagglutination inhibition (HI) titers against A/PR/8/34 were positive at 5 weeks, 9 weeks and 12 weeks after the first injection of V1J-PR-HA DNA. Results are shown below in Table 1:

TABLE 1

HI ANTIBODY TITER OF RHESUS MONKEYS RECEIVING V1J-PR-HA DNA

RHESUS #	DOSE	HI ANTIBODY TITER AT WEEK #				
		PRE	3 WK	5 WK	9 WK	12 WK
88-01	1 MG	<10	<10	320	320	320
88-0200		<10	<10	<10	40	40
88-021	100 UG	<10	<10	<10	40	20
90-026		<10	<10	20	20	40
88-084	10 UG	<10	20	40	20	10
90-028		<10	<10	20	<10	<10

EXAMPLE 11

Polynucleotide vaccine studies in ferrets

1. A study of polynucleotide vaccination in ferrets was initiated with the purpose of determining whether animals could be protected from influenza A infection by immunization with genes encoding either the HA (a surface protein capable of inducing strain-specific neutralizing antibody) or the interal protein NP, NS1, PB1, M (thought to induce a cell-mediated immune response that would be strain-independent). Animals were injected with DNA encoding the various influenza genes in our V1J-vector as shown:

TABLE 11-1

Group	Construct	Dose	No. Animals Immunized	Chall. H1N1	Chall. H3N2
1	V1J-HA	1000 mg	16	8	8
2	V1J-NP	1000 mg	16	8	8
3	V1J-NP+NS1+ PB1+PB2+M	2000 mg total	16	8	8
4	V1J-HA+NP+ NS1+PB1+ PB2+M	2000 mg total	16	8	8
5	V1J-	1000 mg	16	8	8
6	None	None	10	5	5
Total Animals			90	45	45

2. On days 22 and 43 postimmunization, serum was collected from the immunized animals and assayed for neutralizing (hemagglutination inhibiting-HI) antibodies and for antibodies to nucleoprotein (NP) by ELISA. Animals that had been injected with DNA expressed antibodies to the corresponding genes. These are reflected in the attached Figures 10, 11, and 16.

3. On Day 128, selected immunized animals were challenged with 1200 TCID50 of Influenza A/HK/68. This strain is heterologous to the A/PR/8/34 strain that was the source of the coding sequences used to immunize and therefore protection indicates immunity based on cell-mediated, strain-independent immune mechanisms. As shown in the

attached Figure 12, a statistically significant reduction in viral shedding compared to controls was seen in animals immunized with DNA encoding internal proteins, confirming that polynucleotide immunization in ferrets is capable of eliciting an immune response and that such responses are protective.

4. A homologous challenge using A/PR/8/34 is similarly tested and the protective efficacy of neutralizing antibody induced by polynucleotide vaccination is demonstrated similarly.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Dwarki, Varavani J
Liu, Margaret A
Montgomery, Donna L
Parker, Suezanne E
Shiver, John W
Ulmer, Jeffrey B

(ii) TITLE OF INVENTION: Nucleic Acid Pharmaceuticals

(iii) NUMBER OF SEQUENCES: 31

(iv) CORRESPONDENCE ADDRESS:
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(E) COUNTRY: United States of America
(F) ZIP: 07065

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Bencen, Gerard H
(B) REGISTRATION NUMBER: 35,746
(C) REFERENCE/DOCKET NUMBER: 18972IA

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (908)594-3901
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGTGCACCT CAAGCTGG 18

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCTTTGAGA ATGTTGCACA TTC 23

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGTACAAGAT CTACCATGCT TCTAACCGAG GTC 33

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCACATAGAT CTTCACTTGA ACCGTTGCAT CTGCAC 36

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTATATAAGC AGAGCTCGTT TAG 23

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GTAGCAAAGA TCTAAGGACG GTGACTGCAG 30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GTATGTGTCT GAAAATGAGC GTGGAGATTG GGCTCGCAC 39

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTGCGAGCCC AATCTCCACG CTCATTTTCA GACACATAC 39

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

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Thr Tyr Gln Arg Thr Arg Ala Leu Val
1               5
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(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4432 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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CTATTGGCCA TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG    300
TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT AATCAATTAC    360
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ATTTATCAGG GTTATTGTCT CATGAGCGGA TACATATTTG AATGTATTTA GAAAAATAAA 4320
CAAATAGGGG TTCCGCGCAC ATTTCCCCGA AAAGTGCCAC CTGACGTCTA AGAAACCATT 4380

ATTATCATGA CATTAACCTA TAAAAATAGG CGTATCACGA GGCCCTTTCG TC 4432

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2196 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

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GTTCCCATAG TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA GTATTTACGG    300
TAAACTGCCC ACTTGGCAGT ACATCAAGTG TATCATATGC CAAGTACGCC CCCTATTGAC    360
GTCAATGACG GTAAATGGCC CGCCTGGCAT TATGCCCAGT ACATGACCTT ATGGGACTTT    420
CCTACTTGGC AGTACATCTA CGTATTAGTC ATCGCTATTA CCATGGTGAT GCGGTTTTGG    480
CAGTACATCA ATGGGCGTGG ATAGCGGTTT GACTCACGGG GATTTCCAAG TCTCCACCCC    540
ATTGACGTCA ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC AAAATGTCGT    600
AACAACTCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA GGTCTATATA    660
AGCAGAGCTC GTTTAGTGAA CCGTCAGATC GCCTGGAGAC GCCATCCACG CTGTTTTGAC    720
CTCCATAGAA GACACCGGGA CCGATCCAGC CTCCGCGGCC GGGAACGGTG CATTGGAACG    780
CGGATTCCCC GTGCCAAGAG TGACGTAAGT ACCGCCTATA GAGTCTATAG GCCCACCCCC    840
TTGGCTTCTT ATGCATGCTA TACTGTTTTT GGCTTGGGGT CTATACACCC CCGCTTCCTC    900
ATGTTATAGG TGATGGTATA GCTTAGCCTA TAGGTGTGGG TTATTGACCA TTATTGACCA    960
CTCCCCTATT GGTGACGATA CTTTCCATTA CTAATCCATA ACATGGCTCT TTGCCACAAC   1020
TCTCTTTATT GGCTATATGC CAATACACTG TCCTTCAGAG ACTGACACGG ACTCTGTATT   1080
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TTTACAGGAT GGGGTCTCAT TTATTATTTA CAAATTCACA TATACAACAC CACCGTCCCC   1140
AGTGCCCGCA GTTTTTATTA AACATAACGT GGGATCTCCA CGCGAATCTC GGGTACGTGT   1200
TCCGGACATG GGCTCTTCTC CGGTAGCGGC GGAGCTTCTA CATCCGAGCC CTGCTCCCAT   1260
GCCTCCAGCG ACTCATGGTC GCTCGGCAGC TCCTTGCTCC TAACAGTGGA GGCCAGACTT   1320
AGGCACAGCA CGATGCCCAC CACCACCAGT GTGCCGCACA AGGCCGTGGC GGTAGGGTAT   1380
GTGTCTGAAA ATGAGCTCGG GGAGCGGGCT TGCACCGCTG ACGCATTTGG AAGACTTAAG   1440
GCAGCGGCAG AAGAAGATGC AGGCAGCTGA GTTGTTGTGT TCTGATAAGA GTCAGAGGTA   1500
ACTCCCGTTG CGGTGCTGTT AACGGTGGAG GGCAGTGTAG TCTGAGCAGT ACTCGTTGCT   1560
GCCGCGCGCG CCACCAGACA TAATAGCTGA CAGACTAACA GACTGTTCCT TTCCATGGGT   1620
CTTTTCTGCA GTCACCGTCC TTAGATCTGC TGTGCCTTCT AGTTGCCAGC CATCTGTTGT   1680
TTGCCCCTCC CCCGTGCCTT CCTTGACCCT GGAAGGTGCC ACTCCCACTG TCCTTTCCTA   1740
ATAAAATGAG GAAATTGCAT CGCATTGTCT GAGTAGGTGT CATTCTATTC TGGGGGGTGG   1800
GGTGGGGCAG CACAGCAAGG GGGAGGATTG GGAAGACAAT AGCAGGCATG CTGGGGATGC   1860
GGTGGGCTCT ATGGGTACCC AGGTGCTGAA GAATTGACCC GGTTCCTCCT GGGCCAGAAA   1920
GAAGCAGGCA CATCCCCTTC TCTGTGACAC ACCCTGTCCA CGCCCCTGGT TCTTAGTTCC   1980
AGCCCCACTC ATAGGACACT CATAGCTCAG GAGGGCTCCG CCTTCAATCC CACCCGCTAA   2040
AGTACTTGGA GCGGTCTCTC CCTCCCTCAT CAGCCCACCA AACCAAACCT AGCCTCCAAG   2100
AGTGGAAGGA AATTAAAGCA AGATAGGCTA TTAAGTGCAG AGGGAGAGAA AATGCCTCCA   2160
ACATGTGAGG AAGTAATGAG AGAAATCATA GAATTC                             2196
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GTCACCGTCC TTAGATCAAT TCCAGCAAAA GCAGGGTAGA TAATCACTCA CTGAGTGACA 60

TCAAAATCAT G 71

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACCGTCCTTA GATCAGCTTG GCAAAAGCAG GCAAACCATT TGAATGGATG TCAATCCGAC 60

CTTACTTTTC TTAAAAGTGC CAGCACAAAA TGCTATAAGC ACAACTTTCC CTTATAC 117

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 136 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTCACCGTCC TTAGATCAAT TCCAGCAAAA GCAGGGTGAC AAAAACATAA TGGATCCAAA 60

CACTGTGTCA AGCTTTCAGG TAGATTGCTT TCTTTGGCAT GTCCGCAAAC GAGTTGCAGA 120

CCAAGAACTA GGTGAT 136

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 152 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

TCTGCAGTCA CCGTCCTTAG ATCAGCTTGG AGCAAAAGCA GGGGAAAATA AAAACAACCA 60

AAATGAAGGC AAACCTACTG GTCCTCTTAA GTGCACTTGC AGCTGCAGAT GCAGACACAA 120

TATGTATAGG CTACCATGCG AACAATTCAA CC 152

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 162 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TTTTCTGCAG TCACCGTCCT TAGATCCCGA ATTCCAGCAA AAGCAGGTCA ATTATATTCA 60

ATATGGAAAG AATAAAAGAA CTAAGAAATC TAATGTCGCA GTCTGCCACC CCGGAGATAC 120

TCACAAAAAC CACCGTGGAC CATATGGCCA TAATCAAGAA GT 162

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 122 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
GTCACCGTCC TTAGATCTAC CATGAGTCTT CTAACCGAGG TCGAAACGTA CGTACTCTCT    60
ATCATCCCGT CAGGCCCCCT CAAAGCCGAG ATCGCACAGA GACTTGAAGA GTTGACGGAA   120
GA                                                                  122
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG GAGACGGTCA    60
CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG TCAGGGCGCG TCAGCGGGTG   120
TTGGCGGGTG TCGGGGCTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC   180
ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG   240
CTATTGGCCA TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG   300
TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT AATCAATTAC   360
GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT ACATAACTTA CGGTAAATGG   420
CCCGCCTGGC TGACCGCCCA ACGACCCCCG CCCATTGACG TCAATAATGA CGTATGTTCC   480
CATAGTAACG CCAATAGGGA CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC   540
TGCCCACTTG GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA   600
TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG ACCTTATGGG ACTTTCCTAC   660
TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG GTGATGCGGT TTTGGCAGTA   720
CATCAATGGG CGTGGATAGC GGTTTGACTC ACGGGGATTT CCAAGTCTCC ACCCCATTGA   780
CGTCAATGGG AGTTTGTTTT GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA   840
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CTCCGCCCCA TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG 900
AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT TTGACCTCCA 960
TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA CGGTGCATTG GAACGCGGAT 1020
TCCCCGTGCC AAGAGTGACG TAAGTACCGC CTATAGAGTC TATAGGCCCA CCCCCTTGGC 1080
TTCTTATGCA TGCTATACTG TTTTTGGCTT GGGGTCTATA CACCCCCGCT TCCTCATGTT 1140
ATAGGTGATG GTATAGCTTA GCCTATAGGT GTGGGTTATT GACCATTATT GACCACTCCC 1200
CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC ACAACTCTCT 1260
TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA CACGGACTCT GTATTTTTAC 1320
AGGATGGGGT CTCATTTATT ATTTACAAAT TCACATATAC AACACCACCG TCCCCAGTGC 1380
CCGCAGTTTT TATTAAACAT AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCCGG 1440
ACATGGGCTC TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCCTC 1500
CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA GACTTAGGCA 1560
CAGCACGATG CCCACCACCA CCAGTGTGCC GCACAAGGCC GTGGCGGTAG GGTATGTGTC 1620
TGAAAATGAG CTCGGGGAGC GGGCTTGCAC CGCTGACGCA TTTGGAAGAC TTAAGGCAGC 1680
GGCAGAAGAA GATGCAGGCA GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAACTCC 1740
CGTTGCGGTG CTGTTAACGG TGGAGGGCAG TGTAGTCTGA GCAGTACTCG TTGCTGCCGC 1800
GCGCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA TGGGTCTTTT 1860
CTGCAGTCAC CGTCCTTAGA TCTGCTGTGC CTTCTAGTTG CCAGCCATCT GTTGTTTGCC 1920
CCTCCCCCGT GCCTTCCTTG ACCCTGGAA GTGCCACTCC CACTGTCCTT TCCTAATAAA 1980
ATGAGGAAAT TGCATCGCAT TGTCTGAGTA GGTGTCATTC TATTCTGGGG GGTGGGGTGG 2040
GGCAGCACAG CAAGGGGGAG GATTGGGAAG ACAATAGCAG GCATGCTGGG GATGCGGTGG 2100
GCTCTATGGG TACCCAGGTG CTGAAGAATT GACCCGGTTC CTCCTGGGCC AGAAAGAAGC 2160
AGGCACATCC CCTTCTCTGT GACACACCCT GTCCACGCCC CTGGTTCTTA GTTCCAGCCC 2220
CACTCATAGG ACACTCATAG CTCAGGAGGG CTCCGCCTTC AATCCCACCC GCTAAAGTAC 2280
TTGGAGCGGT CTCTCCCTCC CTCATCAGCC CACCAAACCA AACCTAGCCT CCAAGAGTGG 2340
GAAGAAATTA AAGCAAGATA GGCTATTAAG TGCAGAGGGA GAGAAAATGC CTCCAACATG 2400
TGAGGAAGTA ATGAGAGAAA TCATAGAATT TCTTCCGCTT CCTCGCTCAC TGACTCGCTG 2460
CGCTCGGTCG TTCGGCTGCG GCGAGCGGTA TCAGCTCACT CAAAGGCGGT AATACGGTTA 2520

TCCACAGAAT CAGGGGATAA CGCAGGAAAG AACATGTGAG CAAAAGGCCA GCAAAAGGCC 2580

AGGAACCGTA AAAAGGCCGC GTTGCTGGCG TTTTTCCATA GGCTCCGCCC CCCTGACGAG 2640

CATCACAAAA ATCGACGCTC AAGTCAGAGG TGGCGAAACC CGACAGGACT ATAAAGATAC 2700

CAGGCGTTTC CCCCTGGAAG CTCCCTCGTG CGCTCTCCTG TTCCGACCCT GCCGCTTACC 2760

GGATACCTGT CCGCCTTTCT CCCTTCGGGA AGCGTGGCGC TTTCTCAATG CTCACGCTGT 2820

AGGTATCTCA GTTCGGTGTA GGTCGTTCGC TCCAAGCTGG GCTGTGTGCA CGAACCCCCC 2880

GTTCAGCCCG ACCGCTGCGC CTTATCCGGT AACTATCGTC TTGAGTCCAA CCCGGTAAGA 2940

CACGACTTAT CGCCACTGGC AGCAGCCACT GGTAACAGGA TTAGCAGAGC GAGGTATGTA 3000

GGCGGTGCTA CAGAGTTCTT GAAGTGGTGG CCTAACTACG GCTACACTAG AAGGACAGTA 3060

TTTGGTATCT GCGCTCTGCT GAAGCCAGTT ACCTTCGGAA AAAGAGTTGG TAGCTCTTGA 3120

TCCGGCAAAC AAACCACCGC TGGTAGCGGT GGTTTTTTTG TTTGCAAGCA GCAGATTACG 3180

CGCAGAAAAA AAGGATCTCA AGAAGATCCT TTGATCTTTT CTACGGGGTC TGACGCTCAG 3240

TGGAACGAAA ACTCACGTTA AGGGATTTTG GTCATGAGAT TATCAAAAAG GATCTTCACC 3300

TAGATCCTTT TAAATTAAAA ATGAAGTTTT AAATCAATCT AAAGTATATA TGAGTAAACT 3360

TGGTCTGACA GTTACCAATG CTTAATCAGT GAGGCACCTA TCTCAGCGAT CTGTCTATTT 3420

CGTTCATCCA TAGTTGCCTG ACTCCGGGGG GGGGGGGCGC TGAGGTCTGC CTCGTGAAGA 3480

AGGTGTTGCT GACTCATACC AGGCCTGAAT CGCCCCATCA TCCAGCCAGA AAGTGAGGGA 3540

GCCACGGTTG ATGAGAGCTT TGTTGTAGGT GGACCAGTTG GTGATTTTGA ACTTTTGCTT 3600

TGCCACGGAA CGGTCTGCGT TGTCGGGAAG ATGCGTGATC TGATCCTTCA ACTCAGCAAA 3660

AGTTCGATTT ATTCAACAAA GCCGCCGTCC CGTCAAGTCA GCGTAATGCT CTGCCAGTGT 3720

TACAACCAAT TAACCAATTC TGATTAGAAA AACTCATCGA GCATCAAATG AAACTGCAAT 3780

TTATTCATAT CAGGATTATC AATACCATAT TTTTGAAAAA GCCGTTTCTG TAATGAAGGA 3840

GAAAACTCAC CGAGGCAGTT CCATAGGATG GCAAGATCCT GGTATCGGTC TGCGATTCCG 3900

ACTCGTCCAA CATCAATACA ACCTATTAAT TTCCCCTCGT CAAAAATAAG GTTATCAAGT 3960

GAGAAATCAC CATGAGTGAC GACTGAATCC GGTGAGAATG GCAAAAGCTT ATGCATTTCT 4020

TTCCAGACTT GTTCAACAGG CCAGCCATTA CGCTCGTCAT CAAAATCACT CGCATCAACC 4080

AAACCGTTAT TCATTCGTGA TTGCGCCTGA GCGAGACGAA ATACGCGATC GCTGTTAAAA 4140

GGACAATTAC AAACAGGAAT CGAATGCAAC CGGCGCAGGA ACACTGCCAG CGCATCAACA 4200

ATATTTTCAC CTGAATCAGG ATATTCTTCT AATACCTGGA ATGCTGTTTT CCCGGGGATC 4260

GCAGTGGTGA GTAACCATGC ATCATCAGGA GTACGGATAA AATGCTTGAT GGTCGGAAGA 4320

GGCATAAATT CCGTCAGCCA GTTTAGTCTG ACCATCTCAT CTGTAACATC ATTGGCAACG 4380

CTACCTTTGC CATGTTTCAG AAACAACTCT GGCGCATCGG GCTTCCCATA CAATCGATAG 4440

ATTGTCGCAC CTGATTGCCC GACATTATCG CGAGCCCATT TATACCCATA TAAATCAGCA 4500

TCCATGTTGG AATTTAATCG CGGCCTCGAG CAAGACGTTT CCCGTTGAAT ATGGCTCATA 4560

ACACCCCTTG TATTACTGTT TATGTAAGCA GACAGTTTTA TTGTTCATGA TGATATATTT 4620

TTATCTTGTG CAATGTAACA TCAGAGATTT TGAGACACAA CGTGGCTTTC CCCCCCCCCC 4680

CATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG GATACATATT TGAATGTATT 4740

TAGAAAAATA AACAAATAGG GGTTCCGCGC ACATTTCCCC GAAAAGTGCC ACCTGACGTC 4800

TAAGAAACCA TTATTATCAT GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT 4860

CGTC 4864

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCAGAAGCA GAGCA 15

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 119 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

TCACCGTCCT TAGATCAAGC AGGGTTAATA ATCACTCACT GAGTGACATC AAAATCATGG 60

CGTCCCAAGG CACCAAACGG TCTTATGAAC AGATGGAAAC TGATGGGGAA CGCCAGATT 119

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 67 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GAGGGGCAAA CAACAGATGG CTGGCAACTA GAAGGCACAG CAGATATTTT TTCCTTAATT 60

GTCGTAC 67

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCAGAAGCA CGCAC 15

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCAGAAGCA CAGCA 15

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 48 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CCTTAGATCN NNNNNNNACA ACCAAAATGA AAGCAAAACT ACTAGTCC 48

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCAGATCCTT ATATTTCTGA AATTCTGGTC TCAGAT 36

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

ACCGTCCTTA GATCCAGAAG CAGAGCATTT TCTAATATCC ACAAAATGAA GGCAATAATT 60

GTACTACTCA TGGTAGTAAC ATCCAACGCA GATCGAATCT GC 102

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GGCACAGCAG ATCTTTCAAT AACGTTTCTT TGTAATGGTA AC 42

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTAACAGACT GTTCCTTTCC ATG   23

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGAGTGGCAC CTTCCAGG   18

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

AGCAAAAGCA GG   12

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

A[illegible]AGAA[illegible] [illegible]AG[illegible] 14

WHAT IS CLAIMED IS:

1. A DNA construct encoding an influenza virus gene, wherein the DNA construct is capable of being expressed upon introduction into animal tissues in vivo and generating an immune response against the expressed product of the encoded influenza gene.

2. The DNA of Claim 1 wherein the influenza virus gene encodes nucleoprotein, hemagglutinin, polymerase, matrix, or non-structural human influenza virus gene products.

3. A DNA pharmaceutical which induces anti-human influenza virus neutralizing antibody, influenza virus specific cytotoxic lymphocytes, or protective immune responses upon introduction into animal tissues in vivo, wherein the animal is selected from the group consisting of vertebrates, mammals, primates, and humans.

4. The DNA of Claim 3 wherein the nucleic acid is selected from the DNA:

a) pnRSV-PR-NP,
b) V1-PR-NP,
c) V1J-PR-NP, SEQ. ID:12:,
d) V1J-PR-PB1, SEQ. ID:13:,
e) V1J-PR-NS, SEQ. ID:14:,
f) V1J-PR-HA, SEQ. ID:15:,
g) V1J-PR-PB2, SEQ. ID:16:,
h) V1J-PR-M1, SEQ. ID:17:,
i) V1Jneo-BJ-NP, SEQ. ID:20: and SEQ. ID:21:,
j) V1Jneo-TX-NP, SEQ. ID:24 and SEQ. ID:25: and
k) V1Jneo-PA-HA, SEQ. ID:26: and SEQ. ID:27:.

5. The expression vector V1J, SEQ. ID:10:.

6. The expression vector V1J-neo, SEQ. ID:18:.

7. A method for protecting against infection by human influenza virus which comprises immunization with a prophylactically effective amount of the DNA of Claim 1.

8. A method for protecting against infection by human influenza virus which comprises immunization with a prophylactically effective amount of the DNA of Claim 3.

9. A method for protecting against infection by human influenza virus which comprises immunization with a prophylactically effective amount of the DNA of Claim 4.

10. The method of Claim 7 which comprises direct administration of the DNA into tissue in vivo.

11. The method of Claim 10 wherein the DNA is administered either as naked DNA in a physiologically acceptable solution without a carrier or as a DNA-liposome mixture or as a mixture with an adjuvant.

12. A method for using an influenza virus gene to induce immune responses in vivo which comprises:
a) isolating the gene,
b) linking the gene to regulatory sequences such that the gene is operatively linked to control sequences which, when introduced into a living tissue direct the transcription initiation and subsequent translation of the gene,
c) introducing the gene into a living tissue, and
d) optionally, boosting with additional influenza gene.

13. The method of Claim 12 wherein the influenza virus gene encodes a human influenza virus nucleoprotein, hemagglutinin, matrix, nonstructural, or polymerase gene product.

14. The method of Claim 13 wherein the human influenza virus gene encodes the nucleoprotein, basic polymerase1, nonstructural protein1, hemagglutinin, matrix1, basic polymerase2 of human influenza virus isolate A/PR/8/34, the nucleoprotein of human influenza virus isolate A/Beijing/353/89, the hemagglutinin gene of human influenza virus isolate A/Texas/36/91, or the hemagglutinin gene of human influenza virus isolate B/Panama/46/90.

15. A method for inducing immune responses against infection by strains of influenza virus using an influenza gene encoded by a first influenza virus strain such that the induced immune response protects not only against infection by the first influenza virus strain but also protects against infection by strains heterologous to said first strain, which comprises administering an immunologically effective amount of a nucleic acid which encodes a conserved influenza virus epitope.

16. The method of any of claims 7-15 wherein DNA recipient is a human.

17. A vaccine for inducing immune responses against human influenza virus infection which comprises the DNA of any of claims 1-4 and a pharmaceutically acceptable carrier. ?

TITLE OF THE INVENTION
NUCLEIC ACID PHARMACEUTICALS

ABSTRACT OF THE INVENTION

DNA constructs encoding influenza virus gene products, capable of being expressed upon direct introduction, via injection or otherwise, into animal tissues, are novel prophylactic pharmaceuticals which can provide immune protection against infection by homologous and heterologous strains of influenza virus.

Case No. 18972IA

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
DECLARATION AND POWER OF ATTORNEY

The Honorable Commissioner Of Patents And Trademarks
Washington, D. C. 20231

As a below-named inventor, I hereby declare that I believe I am an original, first and joint inventor along with the other inventors listed below, of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NUCLEIC ACID PHARMACEUTICALS

the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended as indicated above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

Foreign Priority

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate for the same invention having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

				Priority Claimed	
Country	Number	Date Filed	Attorney Docket	Yes	No
Country	Number	Date Filed	Attorney Docket	Yes	No

Prior United States Filing

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application(s) in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Appln. Ser. No.	Filing Date	Status	Attorney Docket
08/032,383	3/18/93	Pending	18972
Appln. Ser. No.	Filing Date	Status	Attorney Docket
Appln. Ser. No.	Filing Date	Status	Attorney Docket

I hereby appoint: **Gerard H. Bencen Reg. No.** 35,746, **Raymond M. Speer, Reg. No.** 26,810 **and Jack L. Tribble Reg. No.** 32,633, respectively and individually as my attorneys with full power of substitution and revocation, to prosecute this application and to transact all business connected therewith. Please address all communications to:

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I hereby declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

CASE No. 189721A

1-00

Full Name of Joint Inventor:	John J. Donnelly	Full Name of Joint Inventor:	Varavani J. Dwarki
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3-00 | 4-00

Full Name of Joint Inventor:	Margaret A. Liu	Full Name of Joint Inventor:	Donna L. Montgomery
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2-00

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5-10

Full Name of Joint Inventor: Suezanne E. Parker

Inventor's Signature: Suezanne E Parker

Date: 6/28/93

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Full Name of Joint Inventor: John W. Shiver

Inventor's Signature:

Date:

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Date:

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P.O. Address:
(If different from above)

Full Name of Joint Inventor:

Inventor's Signature:

Date:

Residence:

Citizenship:
P.O. Address:
(If different from above)

CASE No. 18972IA

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Citizenship:	US	Citizenship:	
P.O. Address: (If different from above)		P.O. Address: (If different from above)	US

7.00

Full Name of Joint Inventor:	Jeffrey B. Ulmer	Full Name of Joint Inventor:	
Inventor's Signature:	[signature]	Inventor's Signature:	
Date:	June 30, 1993	Date:	
Residence:	128 Dolly Circle Chalfont, PA 18914	Residence:	
Citizenship:	Canada	Citizenship:	
P.O. Address: (If different from above)		P.O. Address: (If different from above)	

FIGURE 1

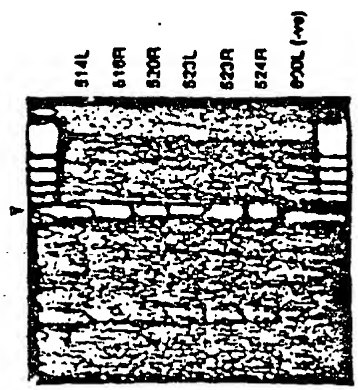


Figure 2

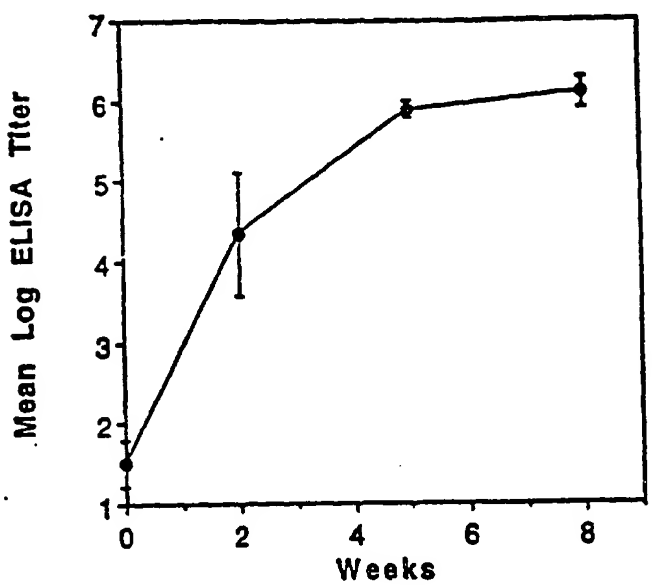
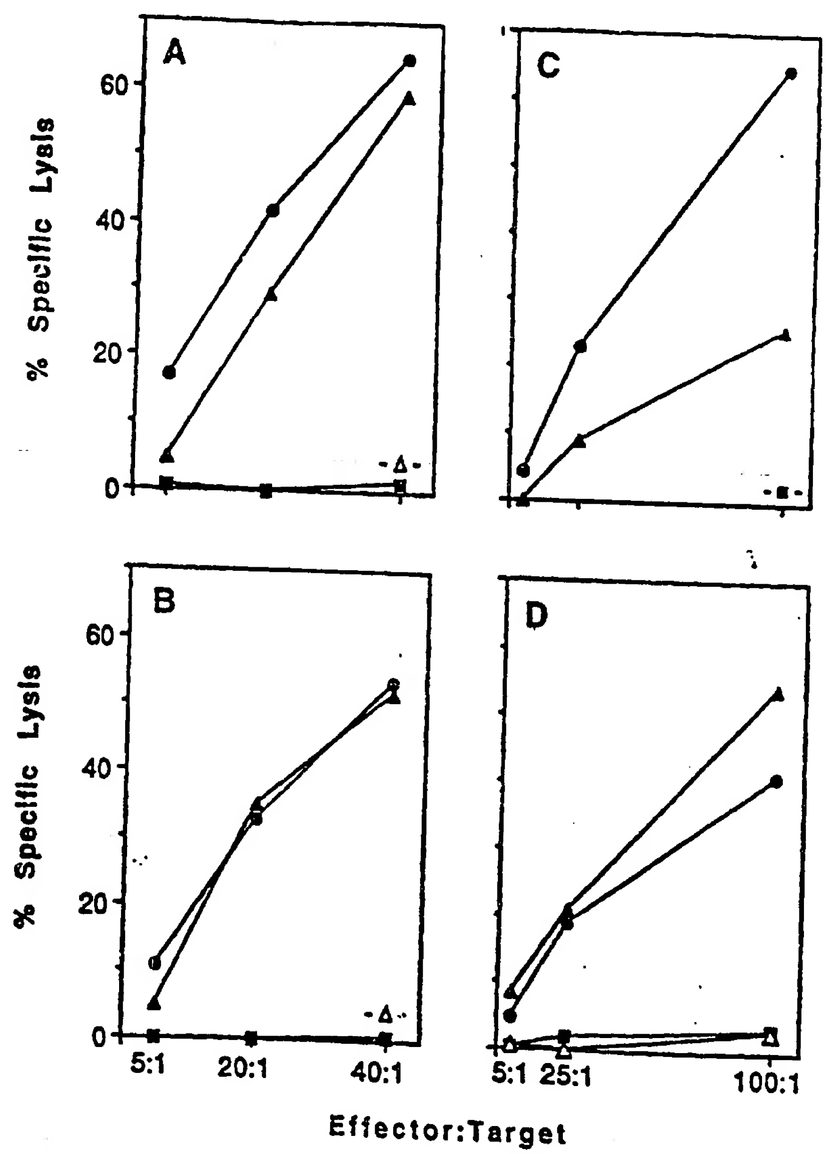
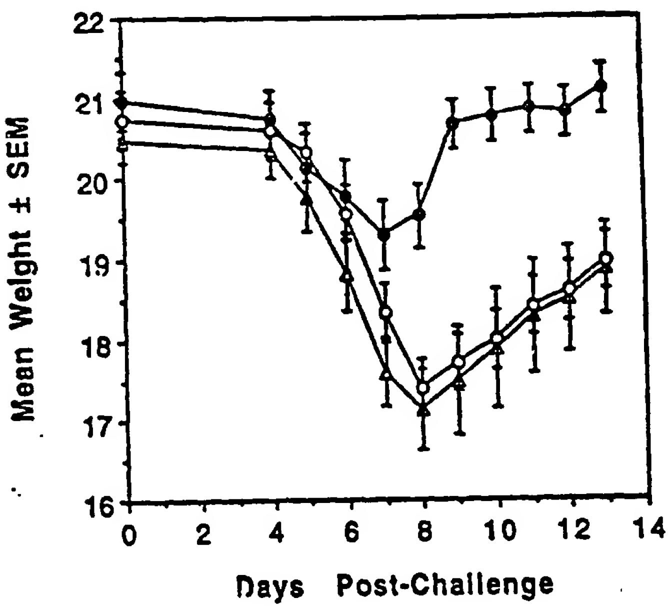


Figure 3



A
B
C
D
% Specific Lysis
% Specific Lysis
60
40
20
0
60
40
20
0
5:1
20:1
40:1
5:1
25:1
100:1
Effector:Target

Figure 4



22
21
20
19
18
17
16
Mean Weight ± SEM
0
2
4
6
8
10
12
14
Days Post-Challenge

Figure 5

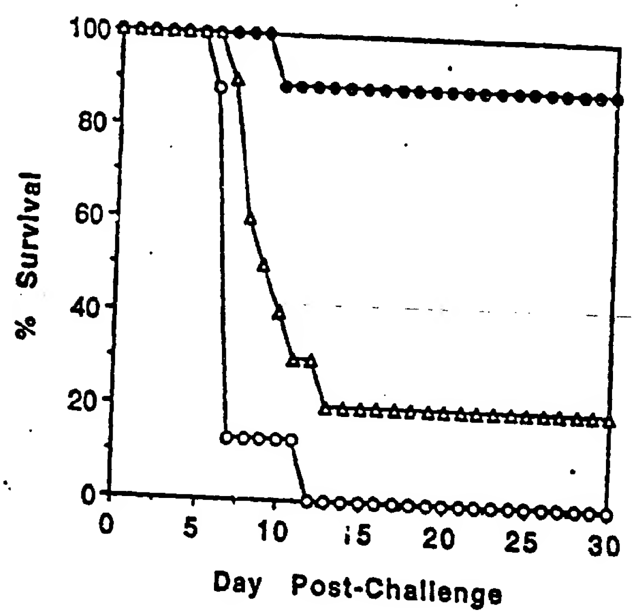


Figure 6: VIJ.Sequence. SEQ. ID 10:

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG
51 GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
101 TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG
151 CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA
201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG CTATTGGCCA
251 TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG
301 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT
351 AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT
401 ACATAACTTA CGGTAAATGG CCCGCCTGGC TGACCGCCCA ACGACCCCCG
451 CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA
501 CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG
551 GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA
601 TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG ACCTTATGGG
651 ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG
701 GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC
751 ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT
801 GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA
851 TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG
901 AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT
951 TTGACCTCCA TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA
1001 CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC
1051 CTATAGAGTC TATAGGCCCA CCCCCTTGGC TTCTTATGCA TGCTATACTG
1101 TTTTTGGCTT GGGGTCTATA CACCCCCGCT TCCTCATGTT ATAGGTGATG
1151 GTATAGCTTA GCCTATAGGT GTGGGTTATT GACCATTATT GACCACTCCC
1201 CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC

Figure 6 (continued, p2/4)

1251 ACAACTCTCT TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA
1301 CACGGACTCT GTATTTTTAC AGGATGGGGT CTCATTTATT ATTTACAAAT
1351 TCACATATAC AACACCACCG TCCCCAGTGC CCGCAGTTTT TATTAAACAT
1401 AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCCGG ACATGGGCTC
1451 TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCCTC
1501 CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA
1551 GACTTAGGCA CAGCACGATG CCCACCACCA CCAGTGTGCC GCACAAGGCC
1601 GTGGCGGTAG GGTATGTGTC TGAAAATGAG CTCGGGGAGC GGGCTTGCAC
1651 CGCTGACGCA TTTGGAAGAC TTAAGGCAGC GGCAGAAGAA GATGCAGGCA
1701 GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAACTCC CGTTGCGGTG
1751 CTGTTAACGG TGGAGGGCAG TGTAGTCTGA GCAGTACTCG TTGCTGCCGC
1801 GCCCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA
1851 TGGGTCTTTT CTGCAGTCAC CGTCCTTAG ATCTGCTGTG CCTTCTAGTT
1901 GCCAGCCATC TGTTGTTTGC CCCTCCCCCG TGCCTTCCTT GACCCTGGAA
1951 GGTGCCACTC CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA
2001 TTGTCTGAGT AGGTGTCATT CTATTCTGGG GGGTGGGGTG GGGCAGCACA
2051 GCAAGGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG
2101 GGCTCTATGG GTACCCAGGT GCTGAAGAAT TGACCCGGTT CCTCCTGGGG
2151 CAGAAAGAAG CAGGCACATC CCCTTCTCTG TGACACACCC TGTCCACGCC
2201 CCTGGTTCTT AGTTCCAGCC CCACTCATAG GACACTCATA GCTCAGGAGG
2251 GCTCCGCCTT CAATCCCACC CGCTAAAGTA CTTGGAGCGG TCTCTCCCTC
2301 CCTCATCAGC CCACCAAACC AAACCTAGCC TCCAAGAGTG GGAAGAAATT
2351 AAAGCAAGAT AGGCTATTAA GTGCAGAGGG AGAGAAAATG CCTCCAACAT
2401 GTGAGGAAGT AATGAGAGAA ATCATAGAAT TTCTTCCGCT TCCTCGCTCA
2451 CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC

Figure 6 (continued, p3/4)

2501 TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA

2551 GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG

2601 CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA

2651 AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA

2701 CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC

2751 TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG

2801 CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG

2851 CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG

2901 CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA

2951 TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT

3001 AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA

3051 GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA

3101 AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG

3151 TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC

3201 AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA

3251 AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC

3301 CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT

3351 ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT

3401 ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCCCGT

3451 CGTGTAGATA ACTACGATAC GGGAGGGCTT ACCATCTGGC CCCAGTGCTG

3501 CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT ATCAGCAATA

3551 AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC

3601 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT

3651 CGCCAGTTAA TAGTTTGCGC AACGTTGTTG CCATTGCTAC AGGCATCGTG

3701 GTGTCACGCT CGTCGTTTGG TATGGCTTCA TTCAGCTCCG GTTCCCAACG

Figure 6 (continued, p4/4)

3751 ATCAAGGCGA GTTACATGAT CCCCCATGTT GTGCAAAAAA GCGGTTAGCT
3801 CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC AGTGTTATCA
3851 CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT
3901 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT
3951 AGTGTATGCG GCGACCGAGT TGCTCTTGCC CGGCGTCAAT ACGGGATAAT
4001 ACCGCGCCAC ATAGCAGAAC TTTAAAAGTG CTCATCATTG GAAAACGTTC
4051 TTCGGGGCGA AAACTCTCAA GGATCTTACC GCTGTTGAGA TCCAGTTCGA
4101 TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT TACTTTCACC
4151 AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG
4201 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT
4251 ATTATTGAAG CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT
4301 GAATGTATTT AGAAAAATAA ACAAATAGGG GTTCCGCGCA CATTTCCCCG
4351 AAAAGTGCCA CCTGACGTCT AAGAAACCAT TATTATCATG ACATTAACCT
4401 ATAAAAATAG GCGTATCACG AGGCCCTTTC GTC

Figure 7: V1Jneo Sequence, SEQ. ID:18.

1 TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG
51 GAGACGGTC. CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG
101 TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG
151 CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA
201 CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGATTGG CTATTGGCCA
251 TTGCATACGT TGTATCCATA TCATAATATG TACATTTATA TTGGCTCATG
301 TCCAACATTA CCGCCATGTT GACATTGATT ATTGACTAGT TATTAATAGT
351 AATCAATTAC GGGGTCATTA GTTCATAGCC CATATATGGA GTTCCGCGTT
401 ACATAACTTA CGGTAAATGG CCCGCCTGGC TGACCGCCCA ACGACCCCCG
451 CCCATTGACG TCAATAATGA CGTATGTTCC CATAGTAACG CCAATAGGGA
501 CTTTCCATTG ACGTCAATGG GTGGAGTATT TACGGTAAAC TGCCCACTTG
551 GCAGTACATC AAGTGTATCA TATGCCAAGT ACGCCCCCTA TTGACGTCAA
601 TGACGGTAAA TGGCCCGCCT GGCATTATGC CCAGTACATG ACCTTATGGG
651 ACTTTCCTAC TTGGCAGTAC ATCTACGTAT TAGTCATCGC TATTACCATG
701 GTGATGCGGT TTTGGCAGTA CATCAATGGG CGTGGATAGC GGTTTGACTC
751 ACGGGGATTT CCAAGTCTCC ACCCCATTGA CGTCAATGGG AGTTTGTTTT
801 GGCACCAAAA TCAACGGGAC TTTCCAAAAT GTCGTAACAA CTCCGCCCCA
851 TTGACGCAAA TGGGCGGTAG GCGTGTACGG TGGGAGGTCT ATATAAGCAG
901 AGCTCGTTTA GTGAACCGTC AGATCGCCTG GAGACGCCAT CCACGCTGTT
951 TTGACCTCCA TAGAAGACAC CGGGACCGAT CCAGCCTCCG CGGCCGGGAA
1001 CGGTGCATTG GAACGCGGAT TCCCCGTGCC AAGAGTGACG TAAGTACCGC
1051 CTATAGAGTC TATAGGCCCA CCCCCTTGGC TTCTTATGCA TGCTATACTG
1101 TTTTTGGCTT GGGGTCTATA CACCCCCGCT TCCTCATGTT ATAGGTGATG
1151 GTATAGCTTA GCCTATAGGT GTGGGTTATT GACCATTATT GACCACTCCC

Figure 7 (continued, p2/4)

1201 CTATTGGTGA CGATACTTTC CATTACTAAT CCATAACATG GCTCTTTGCC
1251 ACAACTCTCT TTATTGGCTA TATGCCAATA CACTGTCCTT CAGAGACTGA
1301 CACGGACTCT GTATTTTTAC AGGATGGGGT CTCATTTATT ATTTACAAAT
1351 TCACATATAC AACACCACCG TCCCCAGTGC CCGCAGTTTT TATTAAACAT
1401 AACGTGGGAT CTCCACGCGA ATCTCGGGTA CGTGTTCCGG ACATGGGCTC
1451 TTCTCCGGTA GCGGCGGAGC TTCTACATCC GAGCCCTGCT CCCATGCCTC
1501 CAGCGACTCA TGGTCGCTCG GCAGCTCCTT GCTCCTAACA GTGGAGGCCA
1551 GACTTAGGCA CAGCACGATG CCCACCACCA CCAGTGTGCC GCACAAGGCC
1601 GTGGCGGTAG GGTATGTGTC TGAAAATGAG CTCGGGGAGC GGGCTTGCAC
1651 CGCTGACGCA TTTGGAAGAC TTAAGGCAGC GGCAGAAGAA GATGCAGGCA
1701 GCTGAGTTGT TGTGTTCTGA TAAGAGTCAG AGGTAACTCC CGTTGCGGTG
1751 CTGTTAACGG TGGAGGGCAG TGTAGTCTGA GCAGTACTCG TTGCTGCCGC
1801 GCGCGCCACC AGACATAATA GCTGACAGAC TAACAGACTG TTCCTTTCCA
1851 TGGGTCTTTT CTGCAGTCAC CGTCCTTAG ATCTGCTGTG CCTTCTAGTT
1901 GCCAGCCATC TGTTGTTTGC CCCTCCCCG TGCCTTCCTT GACCCTGGAA
1951 GGTGCCACTC CCACTGTCCT TTCCTAATAA AATGAGGAAA TTGCATCGCA
2001 TTGTCTGAGT AGGTGTCATT CTATTCTGGG GGGTGGGGTG GGGCAGCACA
2051 GCAAGGGGGA GGATTGGGAA GACAATAGCA GGCATGCTGG GGATGCGGTG
2101 GGCTCTATGG GTACCCAGGT GCTGAAGAAT TGACCCGGTT CCTCCTGGGC
2151 CAGAAAGAAG CAGGCACATC CCCTTCTCTG TGACACACCC TGTCCACGCC
2201 CCTGGTTCTT AGTTCCAGCC CCACTCATAG GACACTCATA GCTCAGGAGG
2251 GCTCCGCCTT CAATCCCACC CGCTAAAGTA CTTGGAGCGG TCTCTCCCTC
2301 CCTCATCAGC CCACCAAACC AAACCTAGCC TCCAAGAGTG GGAAGAAATT
2351 AAAGCAAGAT AGGCTATTAA GTGCAGAGGG AGAGAAAATG CCTCCAACAT
2401 GTGAGGAAGT AATGAGAGAA ATCATAGAAT TTCTTCCGCT TCCTCGCTCA

Figure 7 (continued, p3/4)

```
2451  CTGACTCGCT GCGCTCGGTC GTTCGGCTGC GGCGAGCGGT ATCAGCTCAC
2501  TCAAAGGCGG TAATACGGTT ATCCACAGAA TCAGGGGATA ACGCAGGAAA
2551  GAACATGTGA GCAAAAGGCC AGCAAAAGGC CAGGAACCGT AAAAAGGCCG
2601  CGTTGCTGGC GTTTTTCCAT AGGCTCCGCC CCCCTGACGA GCATCACAAA
2651  AATCGACGCT CAAGTCAGAG GTGGCGAAAC CCGACAGGAC TATAAAGATA
2701  CCAGGCGTTT CCCCCTGGAA GCTCCCTCGT GCGCTCTCCT GTTCCGACCC
2751  TGCCGCTTAC CGGATACCTG TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG
2801  CTTTCTCAAT GCTCACGCTG TAGGTATCTC AGTTCGGTGT AGGTCGTTCG
2851  CTCCAAGCTG GGCTGTGTGC ACGAACCCCC CGTTCAGCCC GACCGCTGCG
2901  CCTTATCCGG TAACTATCGT CTTGAGTCCA ACCCGGTAAG ACACGACTTA
2951  TCGCCACTGG CAGCAGCCAC TGGTAACAGG ATTAGCAGAG CGAGGTATGT
3001  AGGCGGTGCT ACAGAGTTCT TGAAGTGGTG GCCTAACTAC GGCTACACTA
3051  GAAGGACAGT ATTTGGTATC TGCGCTCTGC TGAAGCCAGT TACCTTCGGA
3101  AAAAGAGTTG GTAGCTCTTG ATCCGGCAAA CAAACCACCG CTGGTAGCGG
3151  TGGTTTTTTT GTTTGCAAGC AGCAGATTAC GCGCAGAAAA AAAGGATCTC
3201  AAGAAGATCC TTTGATCTTT TCTACGGGGT CTGACGCTCA GTGGAACGAA
3251  AACTCACGTT AAGGGATTTT GGTCATGAGA TTATCAAAAA GGATCTTCAC
3301  CTAGATCCTT TTAAATTAAA AATGAAGTTT TAAATCAATC TAAAGTATAT
3351  ATGAGTAAAC TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT
3401  ATCTCAGCGA TCTGTCTATT TCGTTCATCC ATAGTTGCCT GACTCCGGGG
3451  GGGGGGGGCG CTGAGGTCTG CCTCGTGAAG AAGGTGTTGC TGACTCATAC
3501  CAGGCCTGAA TCGCCCCATC ATCCAGCCAG AAAGTGAGGG AGCCACGGTT
3551  GATGAGAGCT TTGTTGTAGG TGGACCAGTT GGTGATTTTG AACTTTTGCT
3601  TTGCCACGGA ACGGTCTGCG TTGTCGGGAA GATGCGTGAT CTGATCCTTC
3651  AACTCAGCAA AAGTTCGATT TATTCAACAA AGCCGCCGTC CCGTCAAGTC
```

16.05.94

Figure 7 (continued, p4/4)

3701 AGCGTAATGC TCTGCCAGTG TTACAACCAA TTAACCAATT CTGATTAGAA
3751 AAACTCATCG AGCATCAAAT GAAACTGCAA TTTATTCATA TCAGGATTAT
3801 CAATACCATA TTTTTGAAAA AGCCGTTTCT GTAATGAAGG AGAAAACTCA
3851 CCGAGGCAGT TCCATAGGAT GGCAAGATCC TGGTATCGGT CTGCGATTCC
3901 GACTCGTCCA ACATCAATAC AACCTATTAA TTTCCCCTCG TCAAAAATAA
3951 GGTTATCAAG TGAGAAATCA CCATGAGTGA CGACTGAATC CGGTGAGAAT
4001 GGCAAAAGCT TATGCATTTC TTTCCAGACT TGTTCAACAG GCCAGCCATT
4051 ACGCTCGTCA TCAAAATCAC TCGCATCAAC CAAACCGTTA TTCATTCGTG
4101 ATTGCGCCTG AGCGAGACGA AATACGCGAT CGCTGTTAAA AGGACAATTA
4151 CAAACAGGAA TCGAATGCAA CCGGCGCAGG AACACTGCCA GCGCATCAAC
4201 AATATTTTCA CCTGAATCAG GATATTCTTC TAATACCTGG AATGCTGTTT
4251 TCCCGGGGAT CGCAGTGGTG AGTAACCATG CATCATCAGG AGTACGGATA
4301 AAATGCTTGA TGGTCGGAAG AGGCATAAAT TCCGTCAGCC AGTTTAGTCT
4351 GACCATCTCA TCTGTAACAT CATTGGCAAC GCTACCTTTG CCATGTTTCA
4401 GAAACAACTC TGGCGCATCG GGCTTCCCAT ACAATCGATA GATTGTCGCA
4451 CCTGATTGCC CGACATTATC GCGAGCCCAT TTATACCCAT ATAAATCAGC
4501 ATCCATGTTG GAATTTAATC GCGGCCTCGA GCAAGACGTT TCCCGTTGAA
4551 TATGGCTCAT AACACCCCTT GTATTACTGT TTATGTAAGC AGACAGTTTT
4601 ATTGTTCATG ATGATATATT TTTATCTTGT GCAATGTAAC ATCAGAGATT
4651 TTGAGACACA ACGTGGCTTT CCCCCCCCCC CCATTATTGA AGCATTTATC
4701 AGGGTTATTG TCTCATGAGC GGATACATAT TTGAATGTAT TTAGAAAAAT
4751 AAACAAATAG GGGTTCCGCG CACATTTCCC CGAAAAGTGC CACCTGACGT
4801 CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT AGGCGTATCA
4851 CGAGGCCCTT TCGTC

16.05.94

Figure 8 CMVintaBGH Sequence SEQ ID 11

```
   1 ATTGGCTATT GGCCATTGCA TACGTTGTAT CCATATCATA ATATGTACAT
  51 TTATATTGGC TCATGTCCAA CATTACCGCC ATGTTGACAT TGATTATTGA
 101 CTAGTTATTA ATAGTAATCA ATTACGGGGT CATTAGTTCA TAGCCCATAT
 151 ATGGAGTTCC GCGTTACATA ACTTACGGTA AATGGCCCGC CTGGCTGACC
 201 GCCCAACGAC CCCCGCCCAT TGACGTCAAT AATGACGTAT GTTCCCATAG
 251 TAACGCCAAT AGGGACTTTC CATTGACGTC AATGGGTGGA GTATTTACGG
 301 TAAACTGCCC ACTTGGCAGT ACATCAAGTG TATCATATGC CAAGTACGCC
 351 CCCTATTGAC GTCAATGACG GTAAATGGCC CGCCTGGCAT TATGCCCAGT
 401 ACATGACCTT ATGGGACTTT CCTACTTGGC AGTACATCTA CGTATTAGTC
 451 ATCGCTATTA CCATGGTGAT GCGGTTTTGG CAGTACATCA ATGGGCGTGG
 501 ATAGCGGTTT GACTCACGGG GATTTCCAAG TCTCCACCCC ATTGACGTCA
 551 ATGGGAGTTT GTTTTGGCAC CAAAATCAAC GGGACTTTCC AAAATGTCGT
 601 AACAACTCCG CCCCATTGAC GCAAATGGGC GGTAGGCGTG TACGGTGGGA
 651 GGTCTATATA AGCAGAGCTC GTTTAGTGAA CCGTCAGATC GCCTGGAGAC
 701 GCCATCCACG CTGTTTTGAC CTCCATAGAA GACACCGGGA CCGATCCAGC
 751 CTCCGCGGCC GGGAACGGTG CATTGGAACG CGGATTCCCC GTGCCAAGAG
 801 TGACGTAAGT ACCGCCTATA GAGTCTATAG GCCCACCCCC TTGGCTTCTT
 851 ATGCATGCTA TACTGTTTTT GGCTTGGGGT CTATACACCC CCGCTTCCTC
 901 ATGTTATAGG TGATGGTATA GCTTAGCCTA TAGGTGTGGG TTATTGACCA
 951 TTATTGACCA CTCCCCTATT GGTGACGATA CTTTCCATTA CTAATCCATA
1001 ACATGGCTCT TTGCCACAAC TCTCTTTATT GGCTATATGC CAATACACTG
1051 TCCTTCAGAG ACTGACACGG ACTCTGTATT TTTACAGGAT GGGGTCTCAT
1101 TTATTATTTA CAAATTCACA TATACAACAC CACCGTCCCC AGTGCCCGCA
1151 GTTTTTATTA AACATAACGT GGGATCTCCA CGCGAATCTC GGGTACGTGT
1201 TCCGGACATG GGCTCTTCTC CGGTAGCGGC GGAGCTTCTA CATCCGAGCC
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Figure 8 (continued p2/2)

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1251 CTGCTCCCAT GCCTCCAGCG ACTCATGGTC GCTCGGCAGC TCCTTGCTCC
1301 TAACAGTGGA GGCCAGACTT AGGCACAGCA CGATGCCCAC CACCACCAGT
1351 GTGCCGCACA AGGCCGTGGC GGTAGGGTAT GTGTCTGAAA ATGAGCTCGG
1401 GGAGCGGGCT TGCACCGCTG ACGCATTTGG AAGACTTAAG GCAGCGGCAG
1451 AAGAAGATGC AGGCAGCTGA GTTGTTGTGT TCTGATAAGA GTCAGAGGTA
1501 ACTCCCGTTG CGGTGCTGTT AACGGTGGAG GGCAGTGTAG TCTGAGCAGT
1551 ACTCGTTGCT GCCGCGCGCG CCACCAGACA TAATAGCTGA CAGACTAACA
1601 GACTGTTCCT TTCCATGGGT CTTTTCTGCA GTCACCGTCC TTAGATCTG
1651 CTGTGCCTTC TAGTTGCCAG CCATCTGTTG TTTGCCCCTC CCCCGTGCCT
1701 TCCTTGACCC TGGAAGGTGC CACTCCCACT GTCCTTTCCT AATAAAATGA
1751 GGAAATTGCA TCGCATTGTC TGAGTAGGTG TCATTCTATT CTGGGGGGTG
1801 GGGTGGGGCA GCACAGCAAG GGGGAGGATT GGGAAGACAA TAGCAGGCAT
1851 GCTGGGGATG CGGTGGGCTC TATGGGTACC CAGGTGCTGA AGAATTGACC
1901 CGGTTCCTCC TGGGCCAGAA AGAAGCAGGC ACATCCCCTT CTCTGTGACA
1951 CACCCTGTCC ACGCCCCTGG TTCTTAGTTC CAGCCCCACT CATAGGACAC
2001 TCATAGCTCA GGAGGGCTCC GCCTTCAATC CCACCCGCTA AAGTACTTGG
2051 AGCGGTCTCT CCCTCCCTCA TCAGCCCACC AAACCAAACC TAGCCTCCAA
2101 GAGTGGGAAG AAATTAAAGC AAGATAGGCT ATTAAGTGCA GAGGGAGAGA
2151 AAATGCCTCC AACATGTGAG GAAGTAATGA GAGAAATCAT AGAATTC
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FIGURE 9

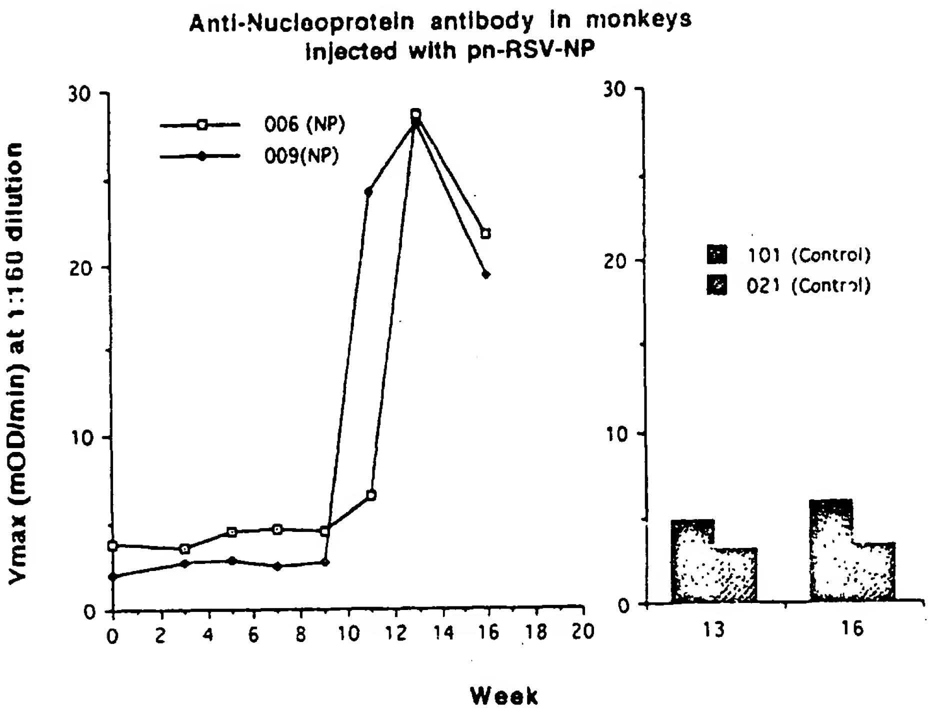

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FIGURE 10

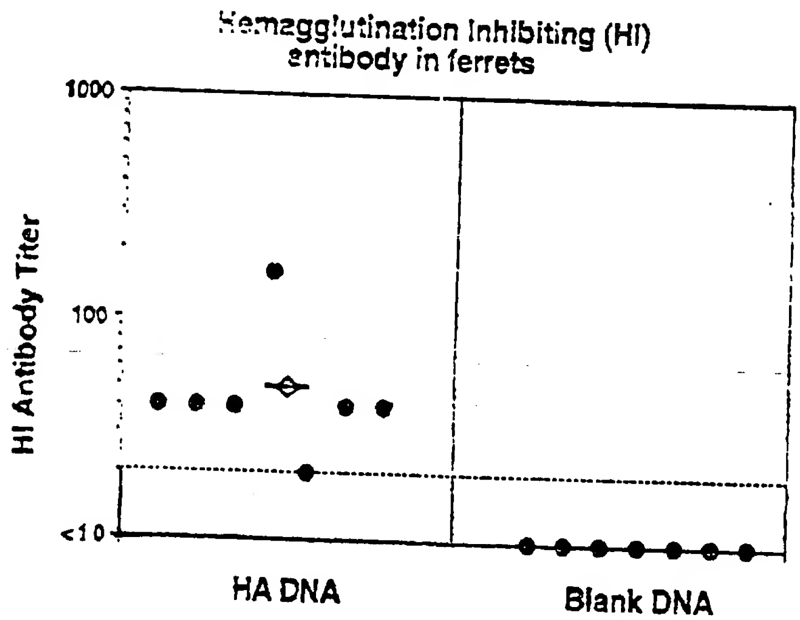

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FIGURE 11

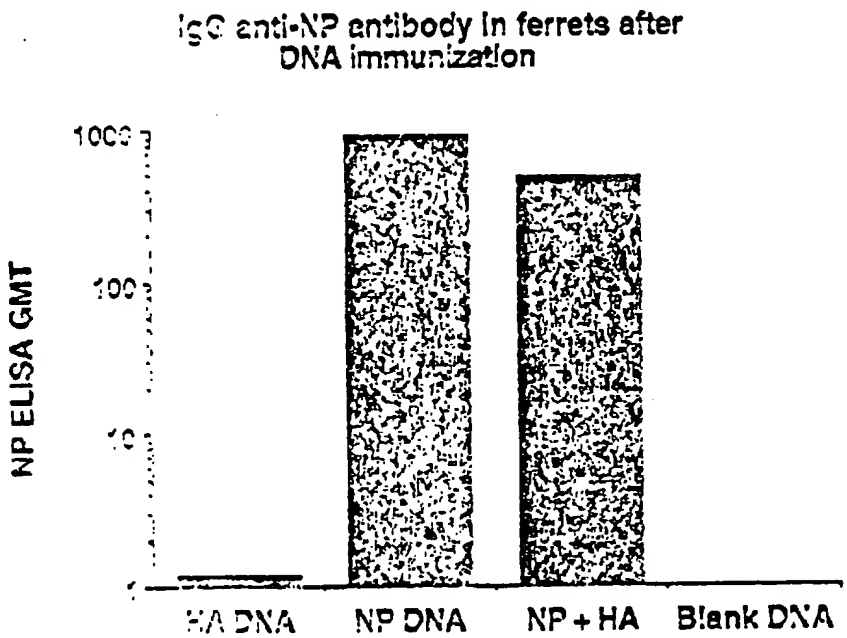

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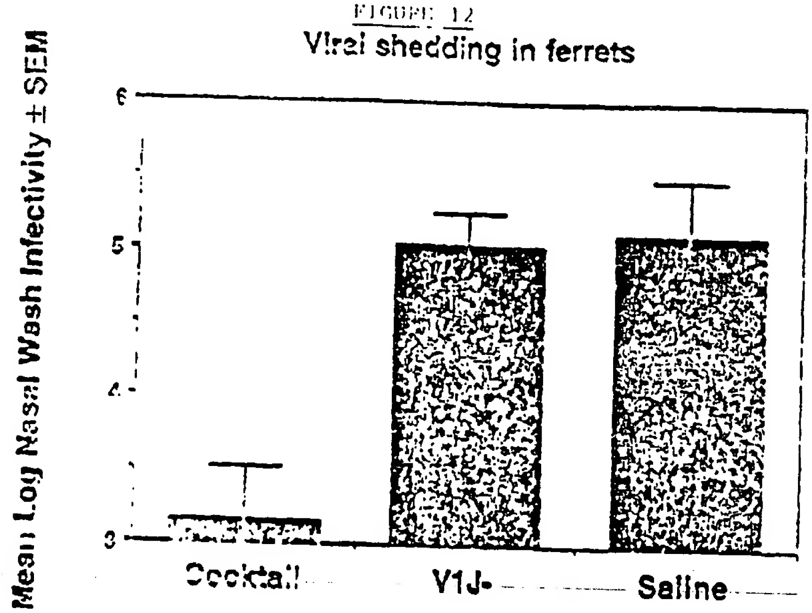
FIGURE 12



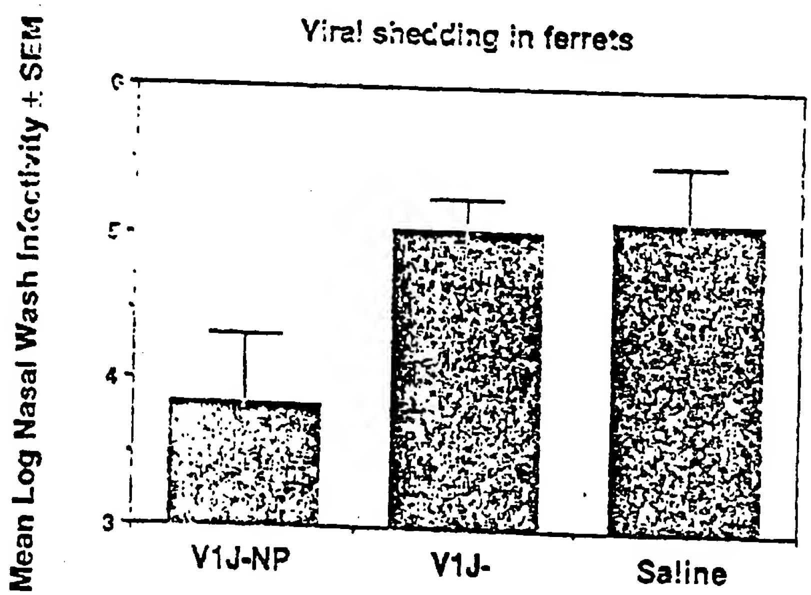


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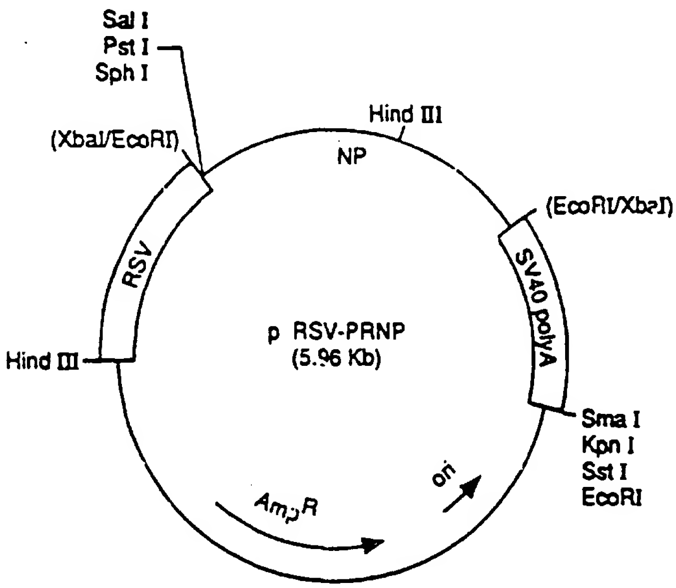
FIGURE 13

PLASMID DNA CONSTRUCTS



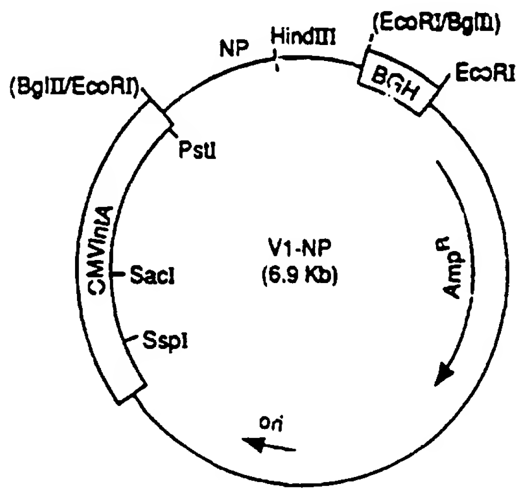


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FIGURE 14

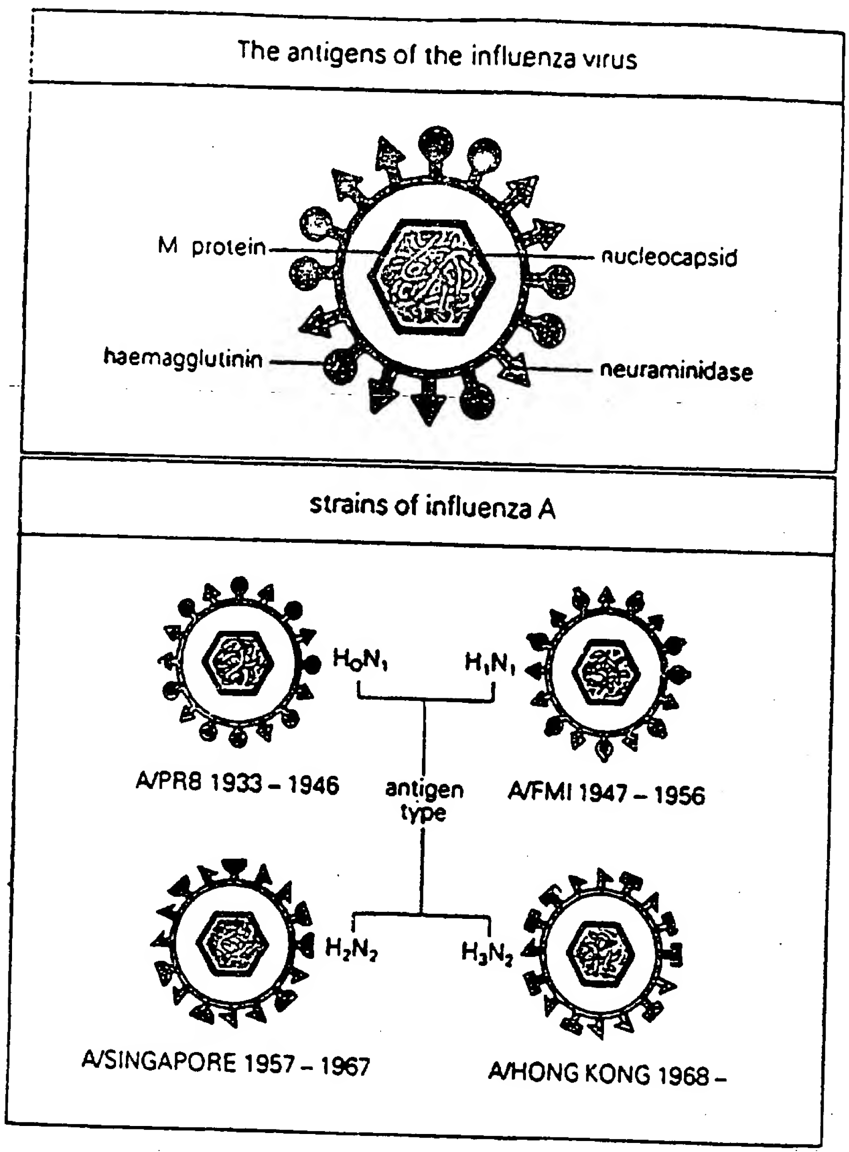

FIGURE 15

INJECTION OF DNA ENCODING A VIRAL PROTEIN GENERATES KILLER T CELLS

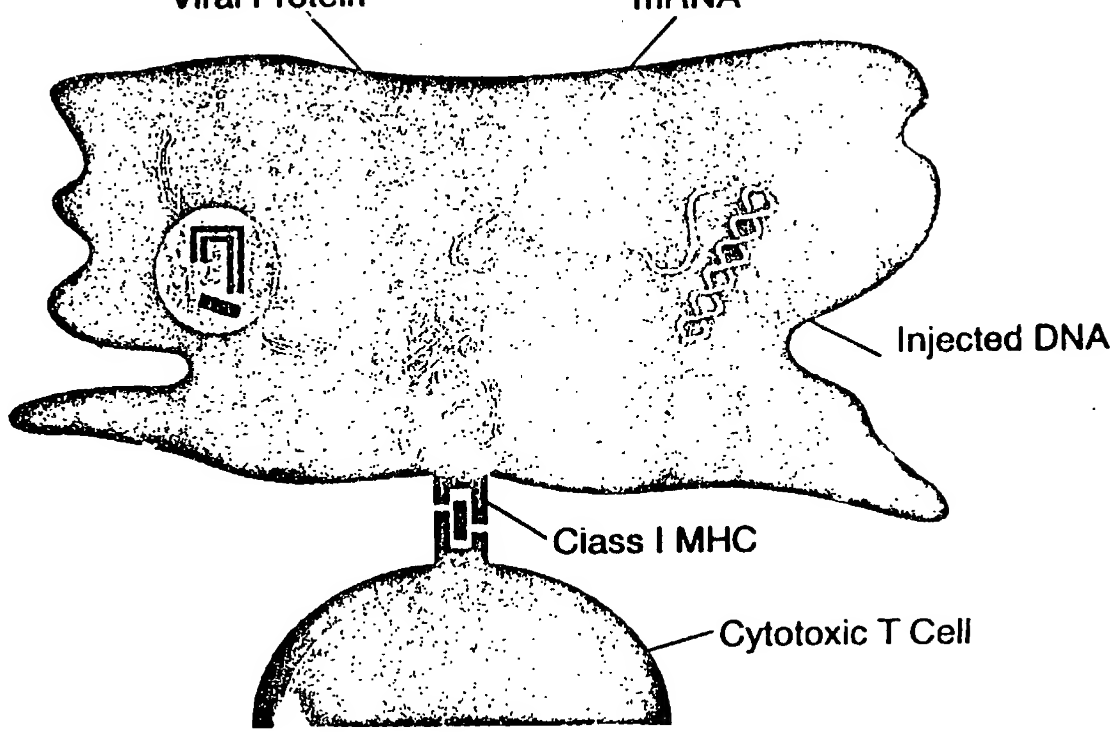

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Figure 16